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# DNA Replication and Genome Maintenance of Human Papillomavirus Type 16 in Mammalian Cells

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# DNA Replication and Genome Maintenance of Human Papillomavirus Type 16 in Mammalian Cells

by

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**DNA Replication and Genome Maintenance of Human Papillomavirus Type 16 in Mammalian Cells**

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Human papillomavirus (HPV) is a major causative agent of cervical cencer and a number of human cancers. The high-risk types can be detected in more than 90% of cervical cancer, of which HPV16 is the most common found. The virus establishes a latent infection in basal epithelial cells in which the viral genomes can be stably maintained as extrachromosomal DNA for decades before the development of cancer. Several attempts have been made in bovine papillomavirus (BPV) to understand how the virus can maintain its genome at constant copy numbers in dividing cells. The viral protein E2 has been proposed to serve as a molecular linker that tethers the viral genome to host chromosomes. However, there is a great deal of dispartities between BPV and HPV with respect to variations of E2 binding pattern, cellular targets, and number of E2 binding sites within their genomes. It is unclear whether HPV utilizes the same maintenance mechanism.

Papillomaviruses utilize two viral factors, E1, a DNA helicase, and E2, a transcriptional activator and auxiliary replication factor for replication and DNA maintenance in host cells. However, previous studies in yeast and findings reported here demonstrated that HPV16 can replicate independently of the viral E1 and E2 proteins. It was also shown that HPV16 possibly contains alternate origin of replication outside the LCR region that relies entirely on cellular replication proteins. In this study, we further identified *cis*-elements and *trans*-acting factors that are required for HPV genome maintenance during persistent infection. Two distinct regions

have been mapped as maintenance elements as they provided DNA stability in mammalian cells in the absence of any viral proteins. We found three nonamer sites (TTAGGGTTA) which resemble telomeric repeats on the *cis*-maintenance elements within the late region and observed altered expression of proteins associated with telomeric repeats in HPV16 immortalized cells. The telomere binding proteins such as telomeric repeat binding factors 1(TRF1),TRF2, TRF2-interacting protein hRAP, the telomere-associated poly (ADP-ribose) polymerase (tankyrase) were previously found on EBV latent origin of replication and contributed to episomal maintenance. These observations motivated us to hypothesize that HPV16 has evolved similar mechanism to maintain its genome in host cells. Using ChIP assay, we found that TRF2, protection of telomere 1 (POT1) and a RecQ helicase WRN bound to the putative binding sites within HPV16 genome. Deletion mutations of TRF binding sites altered the plasmid maintenance activity suggesting the implication of these binding sites as well as the neighboring sequences in HPV life cycle. These results imply that the telomere binding factors are novel cellular factors for HPV16 DNA maintenance. Furthermore, several binding sites for topoisomeraseII (TopoII), centromere binding protein CENP-B, and high mobility group HMG were also predicted in the late region suggesting that HPV maintenance is regulated by multiple cellular factors.

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## TABLE OF CONTENTS

**LIST OF COMMON ABBREAVIATIONS**

| | |
|---|---|
| ARS | Autonomously replicating sequence |
| Bp | base pair |
| BPV | Bovine papillomavirus |
| BLM | Bloom's helicase |
| Brd 4 | Bromodomain 4 |
| BS | Binding site |
| BSA | Bovine serum albumin |
| CEN | Centromere |
| ChIP | Chromatin immunoprecipitation |
| CMV | Cytomegalovirus |
| DNA | Deoxyribonucleic acid |
| EBNA1 | Epstein-Barr virus nuclear antigen 1 |
| EBV | Epstein-Barr virus |
| EDTA | ethylenediamine tetraacetic acid |
| EMSA | Electrophoresis mobility shift assay |
| ECM | Extracellular matrix |

| | |
|---|---|
| EGFP | Enhanced green fluorescent protein |
| FBS | Fetal bovine serum |
| DMEM | Dulbecco's modified eagle's medium |
| DTT | Dithiothreitol |
| HEK | Human embryonic kidney |
| HFK | Human foreskin fibroblast |
| HPV | Human papillomavirus |
| hRAP1 | Human repressor activator protein-1 |
| HSPGs | Heparin sulfate proteoglycans |
| Kb | Kilobase |
| KGM | Keratinocyte growth medium |
| KSHV | Kaposi's sarcoma –associated herpesvirus |
| LCR | Long control region |
| MME | Minichromosome maintenance element |
| NE | Nuclear extract |
| Oct1 | Octamer binding protein 1 |
| ORC | Origin recognition complex |

| | |
|---|---|
| ORF | Open reading frame |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| Poly A | Polyadenylation |
| POT1 | Potection of telomerase 1 |
| PVDF | Polyvinylidene difluoride |
| Rb | Retinoblastoma |
| SDS | Sodium dodacyl sulfate |
| TIN2 | TRF1 interacting factor-2 |
| TBP | Telomere binding protein |
| TBST | Tris-buffered salin Tween-20 |
| TPP1 | Tripeptidyl-peptide 1 |
| TRFs | Telomeric repeat binding factors |
| WRN | Werner's helicase |
| YY1 | Ying yang 1 |

# CHAPTER 1

# LITERATURE REVIEW

***Papillomaviruses: a major cause of human cancers***

Papillomaviruses are nonenveloped, epitheliotrophic DNA viruses that infect board range of animals from birds to mammals, including Humans. They are host-species specific and replicate in a specific type of cutaneous or mucosal epithelial tissues. In humans, papillomaviruses cause a variety of benign lesions such as warts, epithelial cysts, or condylomas, but it is the persistent infection with high risk types that leads to development of malignant carcinomas (Bosch *et al.*, 2002, Schlecht *et al.*, 2001). To date, over 100 different human papillomavirus (HPV) types have been identified (Bernard, 2005, zur Hausen, 2000), of which more than 40 infect the epithelial lining of the mucosal and anogenital tracts. Between 13-18 types are considered as high risk HPV types based on high association with cancer progression (Trottier and Franco, 2006).

The relationship between papillomavirus infections and cervical cancer was first described in 1982 when detection of HPV sequences in human tumors was reported by three different groups (Gissmann *et al.*, 1982, Green *et al.*, 1982, Zachow *et al.*, 1982). It is now well established that HPV is the major causative agent of cervical cancer as high risk HPV DNA is found in greater than 95% of cervical cancers (Munoz *et al.*, 2003, Walboomers *et al.*, 1999). Among those, the most prevalent genotype, HPV 16, was found in greater than 50% of cervical cancers worldwide (Bosch and de Sanjose, 2003, Clifford *et al.*, 2003). HPV infection not only causes the vast majority of cervical cancers, but also is linked to a substantial proportion of other cancers such as anogenital, head and neck, upper respiratory, and even non-melanoma skin cancers (IARC, 1995, Zelkowitz, 2009, zur Hausen, 2009).

HPV-associated diseases cause health problems both on a regional and global scale. There are an estimated 6.2 million new cases of HPV infection reported in the US each year and about 440 million worldwide. The estimated number of deaths due to cervical cancer alone around the world is about a quarter of million per year (National Cancer Institute, 2009) and that HPVs are responsible for the majority of cervical cancers. Although the development of a prophylactic vaccine against the HPVs has been implemented and mandated in certain areas, inequity to access the vaccine in developing countries where cervical cancer is the greatest killer of women is the major barrier to eradication of cervical cancer caused by HPV. Additionally, there are no effective antiviral treatments for curing HPV-associated diseases for those already infected. There are at least 15 oncogenic HPVs, only two of which are included in the current HPV vaccine. Thus, understanding the pathogenesis and viral life cycle is still important

***Life cycle of papillomaviruses***

All papillomaviruses have similar life cycles that are closely linked to the keratinocyte cell differentiation program of the host tissues (Howley and Lowy, 2001). Papillomaviruses establish their life cycle in the stratified epithelium of skin or mucosa, which are composed of multiple layered sheets of keratinocytes with various shapes, in different stages of differentiation. The viral life cycle can be divided into three phases: establishment, maintenance, and amplification (Fig.1.1). Initial infection begins with the access of infectious particles to the mitotically active basal layer cells via minor abrasions or wounds. Papillomaviruses attach to their receptors at the cell surface prior to internalization and migration of their genomes into the nucleus. The identity of the cellular receptors for viral entry is still controversial, however, heparin sulfate proteoglycans (HSPGs) seems to be the best candidate receptors for initial attachment of multiple papillomavirus types (Giroglou *et al.*,

2001, Joyce *et al.*, 1999). Recent work has described the preferential binding of some HPV types to a secreted, keratinocyte-specific extracellular matrix (ECM) protein, laminin 5 (Culp *et al.*, 2006a).he interaction has been shown to be transient (Culp *et al.*, 2006b) and has been proposed to transfer HSPG-bound virions to mitotically active migrating keratinocytes. Other potential targets such as $\alpha$6 integrin (Evander *et al.*, 1997, McMillan *et al.*, 1999) and CD16 (Da Silva *et al.*, 2001) have also been identified as co-receptors for papillomavirus infection.

Viral particles enter the cell through either caveolae or receptor-mediated endocytosis of clathrin-coated pits by a slow process with a 30 minutes (Bousarghin *et al.*, 2003, Day *et al.*, 2003, Hindmarsh and Laimins, 2007, Smith *et al.*, 2007). Virus uncoating may be facilitated by disruption of intracapsomeric disulfide bonds in late endosomes followed by destruction of L1 (Day *et al.*, 2004, Li *et al.*, 1998) and furin cleavage of L2 (Richards *et al.*, 2006). Disassembly of virions allows precleaved L2 to mediate nuclear translocation of the viral DNA to the sub-nuclear promyelocytic leukemia protein (PML) bodies that promote early transcription of papillomaviruses (Day et al., 2004).

After migration of DNA into the nucleus, the viral genome is transiently amplified at a rapid rate to increase the initial genome copy number per cell (Chiang *et al.*, 1992, Ustav and Stenlund, 1991). This initial amplification is followed by stable maintenance phase where the viral DNA is stably maintained at an almost constant copy number (20-100 copies) per infected basal cell (Stubenrauch and Laimins, 1999). Replication of the viral genomes occurs in synchrony with the host cellular DNA during S-phase of the cell cycle through the action of two viral proteins, E1 and E2 (Lambert, 1991, Ustav and Stenlund, 1991). Following viral genome replication and basal cell division, one of the daughter cells migrates away from basal layer and progress in the differentiation program toward maturation of the epithelium. Given the number

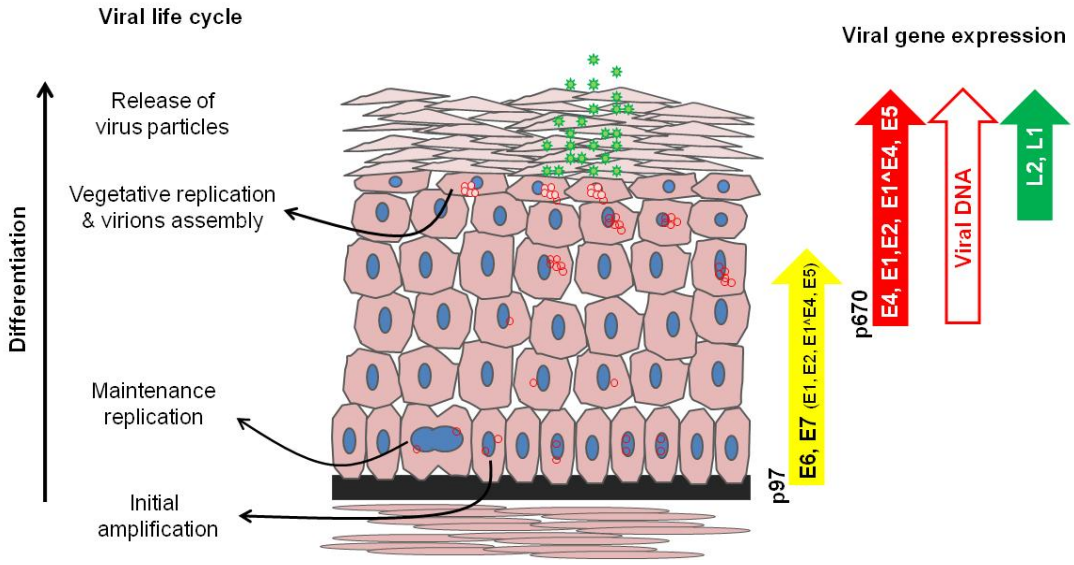


Figure1. 1 The papillomavirus life cycle (left) and the viral gene expression profile (right). The diagram represents stratified epithelium. On the left, the life cycle of papillomaviruses is schematically shown with multiple modes of the viral replication during infection as the infected cells undergo proliferation and differentiation. Virus particles are released by being shed from the superficial surface of epithelium as cornified squames. Infectious particles are able to initiate a new round of life cycle by entering a new basal cell through microabrasions. Viral gene expression is demonstrated on the right with the arrows showing the designated gene that is transcribed in different layers of epithelium. These genes are expressed in a differentiation-dependent manner, and transcription is driven by the early (p97) and late (p670) promoters.

of cell divisions, papillomaviruses require a robust mechanism to ensure a sustained infection by equal segregation of their episomal genomes into the nucleus of daughter cells during cytokinesis.

The virus abolishes the existing constraints on cell cycle progression and retards normal terminal differentiation by the cooperative action of the transforming proteins, E6 and E7 (Chen *et al.*, 1995, Flores *et al.*, 2000). The function of E7 is to disturb the cell-cycle control checkpoints by disruption of negative regulator complex pRb/E2F as well as deregulation of cyclins and cyclin-dependent kinase inhibitors (Funk *et al.*, 1997, Jones *et al.*, 1997, Noya *et al.*, 2001). The viral E6 protein complements this process by mediating p53 degradation, thus, preventing induction of apoptosis in response to unscheduled S-phase entry stimulated by E7 (Doorbar, 2005, Stubenrauch and Laimins, 1999). Induction of S-phase in fully differentiated cell provides a cellular environment that supports high-level viral DNA replication during productive phase. In this stage of the viral life cycle, papillomaviruses switch the replication from once-per-cell cycle manner to the rolling cycle mode, resulting in the subsequent rise of viral genome copy number up to thousands per cell (Dasgupta *et al.*, 1992, Flores and Lambert, 1997).

Differentiation-dependent viral DNA amplification of viral genome in suprabasal cells is proposed to be regulated at transcriptional level (Bedell *et al.*, 1991). The transcription of the viral genes are shifted from the early promoter to the late promoter located within the E7 ORF (Grassmann *et al.*, 1996, Hummel *et al.*, 1992, Klumpp and Laimins, 1999) and this may due to an increase in E1 and E2 protein levels. However, the mechanisms on how keratinocyte differentiation regulates the viral gene expression are not well understood. It is possible that the virus might control viral protein expression in epithelial cells at the posttranscriptional level, depending on cellular factors that are provided by differentiated cells (Zheng and Baker, 2006).

Those mechanisms might involve alternative splicing, polyadenylation or mRNA destabilization (Collier *et al.*, 2002, Zhao *et al.*, 2007, Zhao and Schwartz, 2008).

Regardless of the exact mechanism of gene regulation, the late capsid proteins are synthesized in the upper, terminally differentiated epithelial cells once viral genome amplification has been completed (Ozbun and Meyers, 1998). The restriction of viral DNA amplification and production of viral antigens to the superficial layers of infected tissues inhibits host immune surveillance, resulting in long-term persistent infection. Virions are assembled afterward and it is thought to occur at PML bodies site because the capsid proteins accumulated at this site. Papillomaviruses are non-lytic, and are not released until the virus containing cells reach the epithelial surface and are sloughed from the epidermis. Egress of virions in the upper epithelial layer is achieved by the ability of the viral E4 protein to disturb integrity of keratin organization (Doorbar *et al.*, 1996, Wang *et al.*, 2004).

***Papillomavirus genome structure and gene expression***

Papillomaviruses contain a double-stranded, circular DNA genome of approximately 8 kb in size (Fig.1. 2). The genome can be divided into three major regions: early, late, and a long control region (LCR or upstream regulatory region, URR). The early region contains 8 open reading frames (ORF) designated E1 through E8, which encode nonstructural proteins expressed immediately upon infection (Day et al., 2004). Early transcripts are initiated at a promoter located upstream of the E6 open reading frame (p97 in HPV16) and terminated at a polyadenylation site at the end of the early region ($PolyA_E$). Most of the ORFs in the early region are expressed in the basal or less differentiated cell layers of epithelium.

The late region, lies downstream of the early region and contains two ORFs that are translated into the major (L1) and minor (L2) capsid proteins. The structural proteins are expressed under the regulation of the late promoter (p670 within the E7 ORF in HPV16), which is activated in the more differentiated cells at the upper layer of infected tissues. All ORFs in papillomaviruses are oriented in the same direction and transcribed from one strand.

The LCR, a segment of approximately 800-1,000 Bp located between the early and the late ORFs, has no protein coding function. It bears the replication origin as well as multiple transcription factor binding sites that are important for regulating viral DNA replication and transcription.

Transcription of papillomaviruses is complex, as it involves multiple promoters and polyadenylation sites, extensive alternative splicing with differential patterns of mRNA species, and differentiation specific transcription factors. In high-risk HPVs, viral gene expression is controlled by two major promoters (Chow and Broker, 2007).

The early promoter contains constitutive enhancer elements that control gene expression by cooperative action of several cellular as well as viral transcriptional factors in a cell-type specific manner (Sen *et al.*, 2002). The panel of factors varies among HPV types but some common factors found binding to all HPV genomes include Ap-1 (Kyo *et al.*, 1995, Thierry *et al.*, 1992), Sp-1 (Apt *et al.*, 1996)and TFIID (Tan *et al.*, 1994). Other transcription factors identified include YY1, Oct1, NF1, CBP/p300, CDP, cEBP, glucocorticoid and progesterone receptors, and GRE reviewed in Bernard, 2006 and chow, 2007 (Bernard and Kalantari, 2006, Chow and Broker, 2007). The early primary transcripts terminate at the early polyadenylation (poly$A_E$) and undergo alternative splicing. Postranscriptional processing of the early transcripts

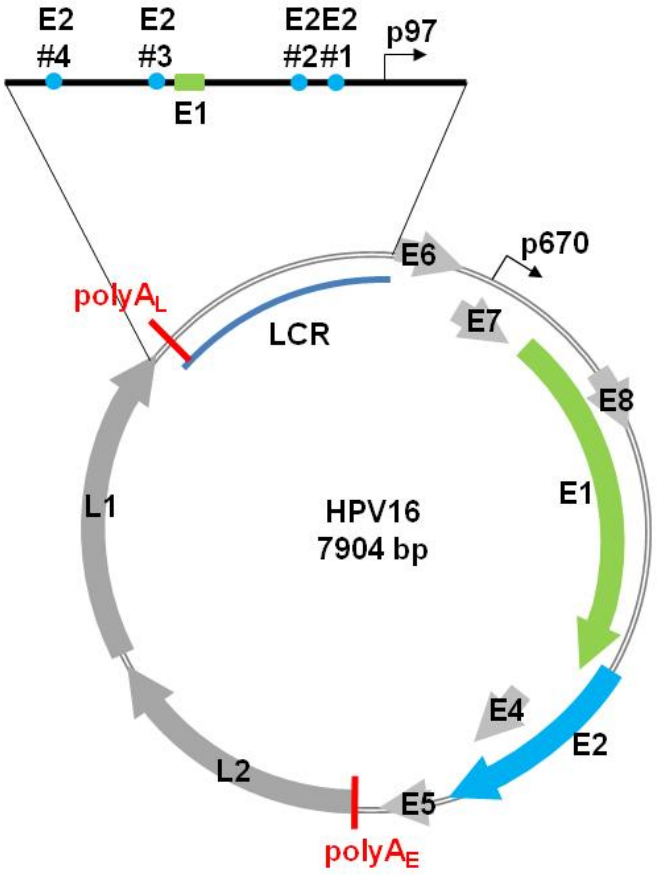


Figure 1. 2 Organization of HPV16 genome. Two coding regions of the early and late genes are indicated as E1-E8 and L1-L2, respectively. The non-coding region, referred to as a long control region (LCR) consists of an early promoter, p97, with the E1 and E2 binding sites. The genome contains two promoters (early; p97, and late promoters; p670) and two polyadenylation sites (polyA$_E$ for early and polyA$_L$ for late polyadenylation sites).

yields at least 14 species of mRNA transcripts that have coding potential for the production of the viral E1, E2, E1^E4, E5, E6, and E7 proteins (Baker and Calef, 1996). The viral E2 protein was first identified as a transcriptional activator that is able to stimulate the activity of the early promoter when it binds to the responsive binding sites in the viral genomes (McBride *et al.*, 1991, Phelps and Howley, 1987). Transcriptional regulation by E2 is more complicated in the presence of shorter forms of the E2 protein, which contain the DNA binding and dimerization domains but lack the transactivation domain. These truncated proteins behave as transcriptional repressors by competing with the full-length E2 for its cognate DNA binding sites and by forming inactive heterodimers with the full-length form (Stubenrauch *et al.*, 2000, Stubenrauch *et al.*, 2001). E2 can also function either as a weak inducer or a strong repressor, depending on the levels of E2 protein (Bouvard *et al.*, 1994). Some reports described further incongruity on the role of E2 in regulation of papillomavirus transcription because high levels of E2 expressed from a surrogate promoter had no transcriptional effect in a cell line containing episomal DNA, but it completely inhibited the transcription in an isogenic cell line bearing integrated viral DNA (Bechtold *et al.*, 2003). Although there are conflicting reports about E2 functions, the family of E2 proteins can have positive as well as negative impacts on viral gene expression (Chin *et al.*, 1989).

It is not well understood how the late promoter is stimulated; but its activation appears to occur upon cellular differentiation and has heterogeneous 5' termini that are located in the E7 gene (Ozbun and Meyers, 1998, Terhune *et al.*, 1999). Late transcripts, driven by the late promoter, either terminated at the early polyadenylation site or the late polyadenylation site (Baker and Calef, 1996). The first group is translated into E1^E4, E5, E1, and E2 whereas the second group encodes the capsid proteins, L1 and L2. Although the cellular factors required for

activation of the late promoter have yet to be identified, it is postulated thatpapillomaviruses modulate the late gene expression by titrating out those factors in highly differentiated cells. Additional minor promoters have been described in multiple papillomaviruses but their biological functions need to be further investigated.

***Papillomavirus DNA replication***

The knowledge regarding papillomavirus replication is originally based on the ability of the bovine papillomavirus type 1 (BPV 1) to infect and transform the mouse fibroblasts cell line (C127) allowing the virus to establish and maintain genomes as extrachromosomal DNA with a constant copy number (Lancaster, 1981, Law *et al.*, 1981). Viral DNA replication observed in these transformed cells is believed to resemble replication occurring in the non-vegetative stage of virus at the basal cells in natural lesions. The BPV system has been used predominantly in the field to study viral replication, hence, it has become a prototype for the papillomaviruses.

Transient transfection plasmids that express E1 and E2 protein in cells that were co-transfected with a reporter plasmid containing the viral origin of replication indicated that initiation of replication is dependent on E1 and E2 proteins (Chiang et al., 1992, Frattini and Laimins, 1994, Ustav and Stenlund, 1991). Replication of papillomavirus origin-containing plasmids has also been recapitulated in vitro with purified E1 and E2 proteins and extracts from mammalian cells (Kuo *et al.*, 1994, Melendy *et al.*, 1995, Muller *et al.*, 1994). These *in vitro* experiments demonstrated that E1, which contains an ATP-dependent helicase, binds specifically to the 18 Bp palindrome recognition sequences located within the viral origin (Chen and Stenlund, 2001, Holt *et al.*, 1994). E1 is responsible for melting the DNA and subsequently unwinding the double helix to allow access of the cellular replication machinery and ultimately,

initiating viral replication (Sedman and Stenlund, 1998). Although the recruitment of host cell replication proteins at the viral replication has not been thoroughly studied, the interaction of E1 with many cellular replication factors such as replication protein A (RPA), topoisomerase and DNA polymerase $\alpha$-primase has been reported (Clower *et al.*, 2006, Conger *et al.*, 1999, Han *et al.*, 1999). Additional cellular factors such as, replication factor C (RFC), proliferating cell nuclear antigen (PCNA), and polymerase $\delta$ may also be involved in viral replication (Kuo et al., 1994, Melendy et al., 1995).

Initiation of papillomavirus replication begins with the cooperative and specific binding of the viral E1 and E2 heterodimers to DNA sequences at the viral origin of replication, which has been mapped to the LCR of the viral genome (Chiang et al., 1992, Sverdrup and Khan, 1994, Ustav *et al.*, 1993, Ustav and Stenlund, 1991). E1 has two different DNA binding domains; the first one is close to the amino-terminus and binds DNA in a sequence-specific manner, the other domain at the carboxyl terminus is the helicase-containing domain that binds DNA with low sequence specificity (Wilson *et al.*, 2002). E2 serves as a specificity factor through the formation of a heteromeric complex with the E1 helicase domain, subsequently leading to inhibition of the non-specific DNA binding of E1 (Bonne-Andrea *et al.*, 1997, Stenlund, 2003). This activity of E2 confers the sequence specific docking of E1 onto the cognate binding site at the origin. In a process that is not well understood, the initiation complex at the origin behaves as a precursor for recruiting a second E1 molecule to form a larger E1 hexamer followed by release of the E2 from the complex. This step appears to occur in an ATP-dependent manner and possibly through the activity of chaperones for displacement of E2 from the complex (Lin *et al.*, 2002).

In general, papillomavirus origins of replication contain an E1 binding site (E1BS), multiple E2 binding sites (E2BSs), and an A/T rich region (Mohr *et al.*, 1990, Ustav et al., 1993, Ustav and Stenlund, 1991). The E1BS is not highly conserved among different HPV strains. It comprises an 18 Bp imperfect palindrome with a consensus ATTGTT , which are present as pairs of sites and separated by three nucleotides from each other (McBride, 2008, Stenlund, 2007). The recognition sites for E2 are very well conserved with the consensus sequence $ACCN_6GGT$ (Androphy *et al.*, 1987, Hawley-Nelson *et al.*, 1988, Hines *et al.*, 1998). The E2BS are found several times in different arrangement and in different types of papillomaviruses. Genital HPVs have four conserved E2BSs located within the LCR whereas the BPV-1 has 17 binding sites which are present inside and outside the LCR (McBride et al., 1991).

Results from studies of various papillomavirus origins reveal that a great deal of flexibility exists among papillomaviruses in terms of the requirement of the E1BS and E2BS as well as the viral E1 and E2 proteins. It has been shown that BPV-1 is capable of replication at high concentrations of E1 in the absence of E2 (Mannik *et al.*, 2002). Other experiments confirmed that E2 is not absolutely required but it could stimulate replication when low levels of E1 are present(Muller et al., 1994, Seo *et al.*, 1993). For BPV-1, it is apparent that a single E2 binding site is sufficient to initiate DNA replication when E1 and E2 are overexpressed (Lu et al., 1993). In contrast to BPV, results from mutation and deletion analyses showed that at least two E2BSs are required for HPVs efficient replication (Remm *et al.*, 1992, Sverdrup and Khan, 1995).

***Stable maintenance replication***

It is thought that maintenance of replication takes place after an initial amplificational phase in the early stage of the viral life cycle. In the maintenance phase, the viral genome is

synthesized at a relatively low copy number and stably maintained by almost equal segregation into the nuclei of daughter cells during the cell divisions. It has been estimated from cell lines that were isolated from clinical lesions and papillomavirus transformed cells that the viral genome replicates in these cells in culture at approximately 50-200 copies per cell (Bedell et al., 1991, Stanley *et al.*, 1989). However, the lower number was estimated in natural host (20 copies per cell) by in situ hybridization of cervical lesions (Evans *et al.*, 2003).

The mechanism of maintenance replication is not still thoroughly understood. It is believed that papillomaviruses duplicate their DNA one time per cell cycle during the S-phase. This type of replication is analogous to Epstein-Barr virus (EBV) latent replication which occurs in synchrony with host cell cycle and is strictly controlled by host cellular factors (Adams, 1987, Yates and Guan, 1991). However, other evidence indicates that papillomavirus replication is confined to the S-phase but in a random-choice control mechanism (Gilbert and Cohen, 1987, Piirsoo *et al.*, 1996). It appears to be more complicated, since a recent report found that while HPV16 replicated one time per cell cycle in W12 cells, HPV31 DNA is replicated by a random-choice mechanism in CIN612 cells. Furthermore, both viruses replicate randomly in an other keratinocyte cell line, NIKS. It was also shown that HPV16 replication was converted to the random-choice mechanism when E1 was expressed at high levels in W12 cells. This suggests that the switch between different modes of replication may be dependent on the level of E1 (Hoffmann *et al.*, 2006). These observations indicate that the mode of papillomavirus replication during the maintenance phase relies on the cell type, and the level of E1. However, some studies suggest that E1 is only needed to establish the viral genome as a nuclear plasmid, but not strictly required for subsequent maintenance replication (Kim and Lambert, 2002). Additionally, experiments in budding yeast *Saccharomyces cerevisiae* have demonstrated that

papillomavirus genomes are able to replicate stably as a plasmid independent of the viral E1 and E2 proteins (Angeletti *et al.*, 2002, Rogers *et al.*, 2008). It is likely that stable maintenance replication relies entirely on the host cell replication factors and this is different from the initial amplification at the early phase of viral life cycle.

The *cis*-elements that contribute to the stable episomal replication have been defined in cells that stably maintain the viral genome extrachormosomally. The origin for maintenance replication was mapped to the same part of the LCR fragment that is used for amplificational replication in dependent of E1 and E2 proteins (Auborn *et al.*, 1994, Flores and Lambert, 1997, Yang and Botchan, 1990). However, there is no clear evidence of the *cis*-elements that are responsible for the E1 independent DNA replication.

***Papillomavirus genome segregration during maintenance phase.***

An interesting feature of papillomaviruses is the ability to maintain the viral genome as an extrachromosomal DNA in the nuclei of infected cells over long period of time (Bedell et al., 1991, Law et al., 1981, Stanley et al., 1989). This property, which is termed maintenance, ensures that the viral DNA is successfully inherited to each daughter cell during cell proliferation. Mechanisms that protect papillomavirus genomes from losing through dissolution of the nuclear membrane upon mitosis or through the nuclear pore have been extensively studied in BPV-1. It has been observed that the virus takes advantage of chromosomal segregation and stability by attachment of the viral DNA to the cellular chromosomes to ensure viral retention. An association with the host chromosomes as a mechanism for viral DNA maintenance has also been described in other latent viruses such as EBV (Harris *et al.*, 1985, Yates *et al.*, 1984) and kaposi's sarcoma virus (KSHV) (Skalsky *et al.*, 2007). They both encode

for site-specific DNA binding proteins that recognize repetitive DNA motifs near the viral origins of replication and attach to the chromosomes during mitosis through cellular proteins.

Several studies in BPV-1 have shown that this process is mediated by the viral E2 protein through physical association between the viral genome and the chromosomes (Ilves *et al.*, 1999, Lehman and Botchan, 1998, Piirsoo et al., 1996, Skiadopoulos and McBride, 1998). E2 serves as a molecular linker that binds specifically to cognate sequences at the LCR and tethers the viral genome to the chromosome via a cellular protein. The best characterized cellular target of E2, to date, is the cellular protein, bromodomain 4 (Brd4) which is a double bromodomain protein that binds to acetylated histone tails of histones H3 and H4 (Baxter *et al.*, 2005, McPhillips *et al.*, 2005, You *et al.*, 2004). However, Brd4 complexed with BPV-1 E2 has been implicated in transactivation function of E2 (Schweiger *et al.*, 2006). It is not clear whether Brd4 provides stability of the viral genome via direct interaction with E2 to link the genome to mitotic chromosome or by enhancing the E2 activity to increase the viral DNA concentration. Recently, multiple chromosomal targets other than Brd4 have been proposed as tethering factors for papillomavirus DNA maintenance. These are chromosome loss-related protein 1 (ChlR1) for BPV-1 and HPV16 (Parish *et al.*, 2006), rDNA for HPV8 (Poddar *et al.*, 2009), and the mitotic spindle for HPV11 (Van Tine *et al.*, 2004).

While the concept of an association between the host chromosome and E2 protein seems to be well established in BPV-1, very little is known about this for HPVs. There are a number of reasons that the BPV-1 maintenance model may not fit HPVs. Firstly, other experiments have failed to support association between HPV E2 and the mitotic chromosome. Some HPV E2 proteins (HPV8, HPV31, HPV16, HPV11) are found colocalized with the chromosomes but there is a great deal of variation with respect to association efficacy, spatio-

temporal pattern of binding as well as cellular partners that E2 interacts with (McBride *et al.*, 2006, McPhillips *et al.*, 2006, Poddar et al., 2009). Further observations demonstrated that at least 10 E2BSs are required for BPV-1 DNA maintenance (Piirsoo et al., 1996, Silla *et al.*, 2005), yet most genital HPVs have only 4 E2BSs. Thus, it is not clear whether this would be sufficient to confer the same stability to the HPVs genomes.

It is possible that these viruses use different cellular targets or viral factors to achieve stable genome maintenance. Several studies in HPV16 have found *cis*-elements that could be important for the viral genome stability. One potential candidate *cis*-element is a nuclear matrix attachment region (MAR) which is mapped in five regions in HPV16 genome *in vivo* (Tan *et al.*, 1998). Most of these are located outside the LCR. It is hypothesized that these elements are responsible for anchoring the viral genome to nuclear matrix, hence, maintain the genome during cell division. Other and our groups have identified a set of *cis*-elements that can autonomously replicate and persist in yeast in the absence of E1 and E2 proteins (Angeletti et al., 2002, Kim *et al.*, 2005, Rogers et al., 2008). Altogether, these observations imply that HPVs employ an alternative mechanism for genome maintenance, potentially relying on cellular factors to a greater extent.

***Telomere related factors and cancer progression***

Induction of telomerase activity by the E6 protein has been well established in high risk HPV (HR HPV) infected epithelial cells (Howie *et al.*, 2009, Klingelhutz *et al.*, 1996) and this process is considered as a critical step for avoidance of cellular senescence that ultimately leads to cancer progression.

Telomerase is over-expressed and activated in most cancers. It maintains telomere length at the ends of linear chromosomes in eukaryotes, hence, provides telomere integrity by preventing telomere erosion that occurs in normal cell during replication. The telomere length maintenance mechanism is believed to enable cells to escape from the normal limitations of proliferative capacity and to become immortalized. However, several studies have demonstrated that regulation of telomere integrity and homeostasis is more complex than can be explained by telomerase activity alone (Gilson and Geli, 2007, Masutomi *et al.*, 2003). This process involves cooperative functions of telomerase and telomere-binding proteins (TBPs) reviewed by Smogorzewska and de Lange (Smogorzewska and de Lange, 2004).

The TBPs are composed of six core proteins that are DNA-binding proteins: telomeric repeat binding factor TRF1, TRF2 and protection of telomere 1(POT1), plus three bridging or adaptor proteins: TRF2-interacting factor RAP1, TRF1-interacting factor TIN2 and tripeptidyl peptide1 (TPP1) (Kanoh and Ishikawa, 2003, Songyang and Liu, 2006). The complex formed by these components, referred to as shelterin or telosome, forms a loop structure that masks the chromosome terminus, preventing it from being recognized as a double-strand DNA break. Apart from the protective activity, dynamic interaction among shelterin subunits and coordinated interplay of these telomeric proteins with telomerase are involved in telomere length regulation in mammalian cells. On the basis of known interaction among the six proteins, TRF1 serves as a negative regulator that controls accessibility of telomerase through its interaction with TIN2 and POT1 (Liu *et al.*, 2004). TRF2, incorporated with POT1, can form a loop structure that plays an essential role in telomere end protection (Stansel *et al.*, 2001, Yang *et al.*, 2005). POT1 binds selectively to the G-rich single stranded of telomeric overhang, protecting the end (Hockemeyer *et al.*, 2006) and limiting telomerase access to the substrate

(Kelleher *et al.*, 2005). However, POT1acts in an opposite fashion when associated with TPP1 to promote telomerase activity (Wang *et al.*, 2007, Xin *et al.*, 2007). TIN1 and TPP1 are the key adaptor proteins that bridge all components to promote the assembly of shelterin (O'Connor *et al.*, 2006). In addition to the telomere length regulation mediated through subcomplexes among shelterin subunits, the TRF1 and TRF2 proteins are able to directly bind double stranded telomeric repeats and then recruit different proteins that are involved in cell cycle, DNA damage, and repair response (Zhu *et al.*, 2000). Thus, these proteins may have different roles in different cellular pathways. Although much evidence supports the existence of subcomplexes that form within shelterin or with other additional proteins, the functional differences among the subcomplexes are presently unclear.

Several studies have reported a wide variety of TBPs levels in many types of cancers compared to normal cells, reviewed by Cookson and Laughton (Cookson and Laughton, 2009). The pattern of change in TBP levels in different cancer types have been investigated. For example, TRF2 and POT1 are up-regulated in breast cancer cell lines, whereas they may be down-regulated in other cell lines. TRF2 expression increases in early stage gastric tumors, but decreases in advanced stages of cancer in separate studies. Contrary to gastric cancer, in hepatocellular cancer levels of TRF1, TRF2, and TIN2 gradually increase according to progression tumorogenesis. Yet, studies in lung, brain and liver cancers showed no change in TBPs levels compared to those in normal tissue counterparts. The differences in relative TBPs levels appear to vary according to the particular cancer type, stage, grade and genetic context. Although the expression of TBPs in cancer is unpredictable based on our current level of knowledge, there is speculation that their expression is crucial for cancer progression through telomere homeostasis. It has been hypothesized that levels of TBPs modulate the sensitivity and

responsiveness of cancers to anticancer therapy that target telomere maintenance. Therefore, TBPs expression in cancer is a potential candidate for anticancer drug development.

***Telomere-related factors and a role in viral maintenance***

It has been well established that telomerase is elevated in HR HPV infected cells and telomerase activation was also observed in cervical keratinocytes containing stably episomal HPV16 DNA. Although little is known about the relationship between telomerase and levels of TBPs in HPV infected cells, it was evidenced that POT1 level was higher in several cervical cancer cell lines compared to normal human cell lines. Since a balance between telomerase, telomere length, and TBPs are key factors to maintain telomeric integrity, it is possible that relative changes in the key mediators might modulate expression of the others in the complex. However, whether telomerase or TBPs influence the HPV life cycle or viral persistence remains to be elucidated.

As mentioned earlier, latent viruses (EBV, KSHV, and HPV) employ similar strategy to maintain their genomes in host cells during persistent infection. The capability to retain the viral genome during the maintenance phase in infected cells by these viruses is related to their long-term roles in host pathogenesis and cancer development. Studies in EBV maintenance/latency revealed that TBPs (TRF1, TRF2, and hRAP1) interact with the EBV's latent origin of plasmid replication (OriP). TRF2 associates with the viral EBNA1 protein and origin recognition complex1 (ORC1), subsequently recruits cellular replicative machinery to initiate DNA synthesis, and duplicate consequently the viral genome (Atanasiu *et al.*, 2006, Deng *et al.*, 2003, Lindner and Sugden, 2007). Moreover, TRF2 contributes to EBV maintenance (Deng *et al.*, 2005, Deng *et al.*, 2002). Experiments in budding and fission yeasts also showed that telomere

repeat sequences and telomere binding protein (RAP1; homolog to TRF1 in Humans) can dramatically improve the segregation of unstable circular plasmids (Longtine *et al.*, 1992). Taken together, it is likely that TBPs may be involved in stable episomal DNA replication and viral genome maintenance in host cells.

Our recent studies in yeast and mammalian cells led us to identify E2-independent *cis*-acting maintenance elements that contain four TRF binding sites within the HPV16 genome (Kim *et al.*, 2005, Pittayakhajonwut and Angeletti, 2008, Rogers et al., 2008). Additional evidence from other laboratory showed that the viral E6 and E7 oncoproteins are important for maintenance of the HPV episomal genome (Thomas *et al.*, 1999). Disturbance of TBPs levels, which could be an important outcome of telomerase induction by E6, and the contribution of TRF1 and TRF2 in EBV genome maintenance cast speculation that TBPs may confer the episomal maintenance of HPV genome during persistent infection.

The knowledge acquired through this study will provide insight into the connection between telomerase, TBPs, and HPV genome maintenance during latency. Since long-term persistence of HPVs is important for cancer pathogenesis, understanding how HPV retains its genome, and what regulates its maintenance will give us novel candidates for antiviral agents. This may lead to development of novel therapeutics that could disrupt the tethering of the viral genome to mitotic chromosome, thus, destroying persistent HPV infection in individuals already infected. Aside from the attractive potential to develop anticancer treatment, HPV *cis*-and *trans*-elements required for HPV persistence can be applied for gene therapy vectors that will stably replicate in dividing cells as extrachromosomal DNA. The first generation vectors consisting of the early genome of BPV-1 were not broadly accepted because they transformed cells and had a limited host range (Waldenstrom *et al.*, 1992). HPV-based vectors have been

constructed and are effective in human cell lines and in lungs of mice (Gadi *et al.*, 1999, Sverdrup *et al.*, 1999). More detailed knowledge of HPV replication and genome maintenance should open up better opportunities to develop more efficient vectors as well as greater antiviral agents with fewer undesirable side effect.

# CHAPTER 2

# Viral Trans-factor Independent Replication of Human papillomavirus Genomes

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**ABSTRACT**

Papillomaviruses (PVs) establish a persistent infection in the proliferating basal cells of the epithelium. The viral genome is replicated and maintained as a low-copy nuclear plasmid in basal keratinocytes. Bovine and human papillomaviruses (BPV and HPV) are known to utilize two viral proteins; E1, a DNA helicase, and E2, a transcription factor, which have been considered essential for

viral DNA replication. However, growing evidence suggests that E1 and E2 are not entirely essential for stable replication of HPV. Here we report that Multiple HPV16 mutants, lacking either or both E1 and E2 open reading frame (ORFs), still support extrachromosomal replication. Our data clearly indicate that HPV16 has a mode of replication, independent of viral trans-factors, E1 and E2, which is acheived by origin activity located outside of the LCR.

**INTRODUCTION**

Papillomaviruses (PVs) infect the basal layers of epithelial cells and maintain their genomes at constant, but relatively low-copy number in basal epithelial cells. These viruses replicate their genomes as nuclear plasmids in their natural mammalian host cells. Understanding of Human papillomaviruse (HPV) replication has lagged behind that of other DNA viruses due to the need for development of efficient cell culture systems (Durst *et al.*, 1987, Korman *et al.*, 1987, Stanley, 1994). Most of our knowledge of HPV replication is derived from extensive studies of BPV1 in established rodent cell lines (C127), since BPV1 was found to transform and replicate episomally in these cells. Short-term replication assays were performed in transformed cells, in order to identify the *cis*- and *trans*-elements that were required for replication of PVs (Stenlund, 1996). Using this approach, with BPV1, the early proteins E1 and E2 were found to be required for viral DNA replication (Chiang et al., 1992, Del Vecchio et al., 1992, Ustav and Stenlund, 1991). These viral proteins interact with their cognate binding sites, located within the LCR, referred to as the origin of replication (ori). More detailed analyses have shown that the minimal BPV origin includes multiple E2BSs, an E1BS, and also an A-T rich region (Ustav et al., 1991).

Genetic analysis of HPV11 and 18 transient replication also suggested that both viral proteins E1 and E2, as well as the origin of replication containing one or more E2BS and putative E1BS and A-T rich region, were essential for HPV DNA replication (Chiang et al., 1992, Demeret *et al.*, 1995, Lu et al., 1993, Mungal *et al.*, 1992, Sverdrup and Khan, 1994). Several experiments, including those performed by cell-free DNA replication, revealed that E1 had ATP-dependent helicase activity and recruits DNA polymerase $\alpha$ to the viral ori to initiate replication. Efficient replication also depends on all other DNA replication proteins; DNA polymerase $\delta$ and $\alpha$,

proliferating cell nuclear antigen (PCNA), replication protein A (RPA), and topoisomerases I and II, that are provided by the host cell (Kuo et al., 1994, Melendy et al., 1995).

Although it is apparent that the minimal requirement for HPV replication is analogous to that for BPV1 in transiently transfected cells, HPV16 genomic DNA has been shown to replicate at lower efficiency than other HPVs (Del Vecchio et al., 1992). Furthermore, results from cell free replication with combination of ori and viral proteins showed certain differences in replication between BPV and HPV. Variations in the viral replication efficiency observed with cell extracts from different sources might reflect dissimilar function of HPV with different host cellular replication machinery (Kuo et al., 1994).

Published data that indicated a requirement for E1 and E2 proteins among PVs was mostly from transient transfection assays that were performed in the context of exogenously expressed viral proteins. The concentrations used in those experiments would not reflect physiological levels of the viral proteins in the basal layer of epithelial cells in natural host tissue. Since the viral life cycle is tightly linked to the differentiation status cell in the epithelial compartment, it is concievable that HPVs may have more than one mode of DNA replication, so as to allow fine-tuning. In fact, recent data has shown that an E1-independent mode of replication exists in the viral replication at early stage of viral life cycle (Kim, 2002, Kim and Lambert, 2002). This result is in agreement with previous experiments in *Saccharomyces serevisiae* showing that various HPVs can support viral replication in the absence of both E1 or E2 proteins (Angeletti et al., 2002). Moreover, the *cis*-acting elements required for E1 and E2 - independent replication and maintenance were also mapped outside the LCR region (Kim et al., 2005). These findings lead us to hypothesize that at certain point of the viral life cycle, the HPV

replication may not require both of the viral trans-factors, E1 and E2, but may rely solely on cellular replication machinery.

In this study, we analyzed the requirement for E1 and E2 in HPV16 DNA replication at the early stage of the viral life cycle. Using a short-term replication assay, as previously used for BPV and HPVs (Del Vecchio et al., 1992, Ustav and Stenlund, 1991), we have found that HPV16 is able to replicate independently of viral trans-factors, E1 and E2. We present evidence that HPV16 possesses a distinct origin of replication-activity, located in a region outside of the viral LCR that is not recognized by the viral proteins, E1 and E2. However, replication of HPV16 DNA in the absence of heterologously expressed E1 and E2 proteins is relatively low compared to the E1 and E2-mediated replication. This is consistent with a low, maintenance level of replication. In contrast to previous results from transient transfection and cell free system (Chiang et al., 1992, Ustav and Stenlund, 1991), we observed species and cell-type specificity to HPV16 DNA replication under conditions in which E1 and E2 were omitted.

## MATERIALS AND METHODS

### *Mammalian cells and transfection methods*

American green monkey kidney; Vero, osteoblastoma; U2OS, human embryonic kidney; 293, and human cervical carcinoma; C33A were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate in a 5% $CO_2$ incubator at 37°C. Baby hamster kidney; BHK, was cultured in DMEM supplemented with 5% fetal bovine serum. All transfections were performed with eqimolar amounts of plasmid DNA by the use of Dreamfect reagent (Ozbiosciences). Cells were seeded at the density of 1.5–2 x $10^6$ cells in a 100 mm plate for 16 to 24 h prior to transfection.

***Plasmids and library constructions***

The PA99GFP plasmid contains a full-length HPV16 genome cloned into pUC19 with enhanced green fluorescent protein (EGFP) under the regulation of the cytomegalovirus promoter. This plasmid was modified from pPA99 (Angeletti et al., 2002) by insertion of EGFP gene which was PCR-amplified from pGFP C1 using primers (5' primer: 5'-GCATCCTCGAGGTAATCAATTACGGG-3' and 3' primer: 5'-CGAAGCTTGAGCTCGAGATCTGAG-3') containing XhoI site (underlined) into the XhoI site of pPA99. Deletion mutants designated ΔE1-E2#13, ΔE-E2#15 and ΔE1-E2#18 which are indicated in Fig 2.2A, were obtained from modifications of PA99GFP. The HPV16 E2 deletion mutants were made by cleavage of PA99GFP with SexAI and XcmI. After the removal of SexAI-XcmI fragment, the protruding ends were subsequently repaired by DNA polymerase I (Klenow) and religated. The resultant plasmids were screened for ones that lack the SexAI-XcmI fragment but retain the selectable marker. Three plasmids were selected and subjected to DNA sequencing analysis. The first one had a 3585 bp deletion (nucleotides 339-3924) resulting in removal of the E1 and E2 ORFs referred to as ΔE1-E2#13. The second one, named ΔE1-E2#15, lacked HPV16 sequences between nucleotides 7394-7904 and 1-3924 within the LCR, E6, E7, E1 and E2 ORFs. For the last HPV16 E2 mutant, designated as ΔE1-E2#18, a deletion was created between nucleotides 3661-5240, thus leaving E1 ORF intact but truncating the E2 and L2 ORFs.

The HPV16GFP plasmid contains the entire HPV16 genome in pUCGFP that carries an expression cassette of EGFP gene in a pUC19 backbone. HPV16 mutants designated ΔLCR-E2, ΔE1-E2, and L1 were derived from HPV16GFP as previously described (Pittayakhajonwut and Angeletti, 2008). The mutant ΔLCR was made by deleting a 909-bp fragment between PmlI and BsaBI sites and then religated by T4 DNA ligase. To confirm the deletion, DNA sequencing

analysis was performed afterward by standard procedures. A map of HPV16GFP and its derivative constructs is shown in Fig 2.3A.

***Short-term DNA replication assay.***

Cells were seeded into a 100 mm dish at the density indicated above and transfected with 0.6 pmoles of plasmid DNA using the manufacturer's instructions. The plasmid-containing cells were grown under non-selective conditions for 4 days prior to analysis. Plasmid DNA was isolated using the Hirt method (Hirt, 1967) from equal numbers of cells ($6 \times 10^6 - 1 \times 10^7$ cells). Ten ng of pUC19, referred to as "spiked DNA", was added to each sample prior to cell lysis in order to monitor the efficiency of plasmid isolation from cells and the completeness of subsequent Dpn I digestion. Approximately 10% of each sample was digested with 10 U of a single-cutting enzyme, HindIII, at 37°C for 4 h, (resulting in over 90% completion of HindIII digestion), followed by overnight digestion with 20 U DpnI. Upon digestion, DNA was seperated on a 0.8% agarose gel and then transferred to a nitrocellulose membrane. Blots were hybridized at 42°C overnight with a pUC19 DNA probe radiolabeled by random priming kit (GE Healthcare). Autoradiography was carried out by use of a PhosphoImager (Bio-Rad) and quantified by the Quantity One program (Bio-Rad). Relative replication activities were quantified from a phosphorimage of the Southern blots. The percentages of replicated DNA refer to the ratio of the level of replicated DNA (DpnI-resistant plasmid) over the level of HindIII-linearized plasmid. The numbers were normalized against the level of the spiked DNA in each sample.

**RESULTS**

***Transient replication of HPV16 genomic DNA in different cell lines.***

In order to determine the requirement for viral trans-factors in HPV16 DNA replication, different mammalian cell lines of epithelial or fibroblast lineage were examined for the ability to support transient viral DNA replication. Baby Hamster Kidney cells (BHK), monkey kidney cells (Vero), human osteoblasts (U2OS), human cervical cells (C33A), and adenovirus 5-transformed human kidney epithelial cells (293) were transfected with 5 µg o f a plasmid containing an entire HPV16 genome. At 4 days after transfection, low-molecular-weight DNA was harvested and subjected to Dpn I digestion. Input DNA propagated by $DAM^+$ bacteria is methylated at adenine bases within the GATC sequence, and is thus sensitive to DpnI difestion, whereas DNA replicated in mammalian cells loses the adenine methylation and thus, becomes DpnI-resistant. The replicated DNAs were detected as full-length plasmids by Southern analysis using pUC19 as a probe. Human 293 and C33A demonstrated the ability to support viral DNA replication at a low level, whereas BHK, Vero, and U2OS failed to replicate under the same conditions (Fig 2.1). Variation in viral DNA replication in different cell lines clearly indicates a degree of species and/or cell-type specificity in HPV16 replication. This also reflected the greater reliance of HPV16 on the host replication machinery when E1 and E2 were not exogenously supplemented.

***Analysis of HPV16 E1- and E2-independent mode of replication***

Based on the observation of E1-independent HPV replication in previous studies, in yeast or in mammalian cells, we speculated that HPV16 may not require either E1 and E2 proteins for replication of its genome at a certain stage of the viral life cycle. To investigate the necessity for E1 and E2 in HPV16 DNA replication, HPV16 mutants with deletions that included the E1 and E2 ORFs were generated in the background of the entire viral genome in a pUC19 backbone. The mutants, designated as ΔE1-E2#13 and ΔE1-E2#15 lack E1 and E2 sequences to differing extents, as shown in Fig 2.2A. These deletion mutants were examined for short-term replication using human epithelial

cells, 293 and HPV-negative human cervical cells, C33A that were previously shown to support the replication of wild type HPV16. The transient replication assay most closely mimics the early phase of the HPV life cycle, thus, representing the initial viral DNA replication upon HPV infection. Analysis of short-term replication of HPV16 mutants defines the *cis*- elements required for HPV replication at the establishment phase of the HPV life cycle. Equal amounts of HPV16 mutants (0.6 pmole equivalent) were transfected into 1.5 x $10^6$ cells by the liposome method. To compare replication efficiency, a plasmid containing an entire HPV16 genome was transfected in parallel under the same conditions. Low-molecular-weight DNA was extracted at 4 day post-transfection and analyzed for Dpn I-resistant replicated DNA as described above.

As shown in Fig 2.2 B and C, a mutant with a deletion that included the E1 and E2 ORFs, designated as ΔE1-E2#13 (lanes 2) showed DNA replication at a level comparable to that of the WT HPV16 in either 293 or C33A cells. A similar level of replication was also observed in a mutant, ΔE1-E2#15 (lane 3) that lacks both of E1-E2 coding regions and a part of the LCR, which contains the E1 and E2 binding sites at the origin of replication. These results indicate that the viral proteins, E1 and E2, are not absolutely necessary for the viral DNA replication. Thus, it is likely that HPV16 has sequences outside of the LCR, which behave as an alteranate autonomously replicating sequence (ARS) that is not recognized by the viral *trans*-factors.

To determine if HPV16 carries a distinct origin of replication that functions independently of E1 and E2 proteins, a series of mutants with progressive deletions spanning the LCR to the E1 and E2 ORFs were constructed and tested for ability to replicate in a transient transfection assay. The deletion mutants were created in a context of a whole HPV16 genomic DNA in a pUC19 backbone. The mutant, ΔLCR, contains a deletion (nt 7266 to 7904/1 to 269) in the LCR sequence where E1 and E2 bindings sites are located. In the ΔE1-E2 mutant, the whole E1 and E2 ORFs (nt

269 to 4466) were deleted. The mutant, ΔLCR-E2, contains a combination of deletions in the ΔLCR and ΔLCR-E2 mutants, whereas the L1 mutant contains only a 3'-segment of the L1 ORF (nt 6156 to 7265). The replication activities of these mutants were analyzed in both C33A and 293 cells.

When performed a transient transfection assay, each mutant was found to replicate at a similar level to that of wild type HPV16 (Fig 2.3B and C, lanes 2 to 5), whereas, the plasmid backbone without HPV16 DNA sequences, PUCGFP, did not show any detectable DNA replication in either 293 and C33A cells (Fig 2.3B and C, lane 6). Interestingly, the mutant containing only a partial segment of L1 coding region was able to replicate in both cell lines with efficiency comparable to those of wild type and other mutants. These observations not only confirm the existence of E1 and E2 independent mode of replication in HPV16 but also indicate that the virus has a distinct ARS, outside of the LCR, the activity of which is likely to be mediated only by cellular replication machinery. Our data indicate that this viral-trans-factor independent *cis*-replicating element resides in the late region of HPV16 genome (L2-L1 ORFs).

**DISCUSSION**

HPVs establish their life cycle in the stratified epithelium of skin or mucosa. The virus replicates and amplifies its genome in two different stages, non-productive and productive cycles. In the non-productive stage, the first amplification replication occurs immediately after infection and the viral DNA is stably maintained in the undifferentiated basal cells. Whereas the second amplification replication, which increases the HPV DNA copy number to several hundreds or thousands per cells, takes place after differentiation of the host cell. Based on the knowledge acquired by studies with BPV1, the early step in the PV replication may occur at relatively low levels of E1 and E2 proteins. This amplification is thought to be rapid and transient, after which, the viral

DNA is maintained at low copy number during the maintenance phase and persists in a latent stage.

It would be reasonable to suggest that during maintenance phase, HPV keeps the levels of E1 and E2 and the viral copy number low in the infected basal cells, so as to evade cellular immune surveillance. It is also logical that the virus may employ a different mode of replication, which relies more on cellular replication machinery to stably retain the genome as a nuclear plasmid in the proliferating basal epithelial compartment. The virus replicates once per cell generation in the presence of low-levels of E1, and later converts to random-choice replication, with multiple rounds of viral DNA replication per cell generation, when E1 is highly expressed (Hoffmann et al., 2006). This concept is also consistent with results of previous studies which found that E1 was dispensible for PV replication, under some conditions (Kim and Lambert, 2002).

There are conflicting results concerning the requirement of E2 in replication of PVs. In some studies, E2 ORF has been shown not to be necessary for replication while others have found an absolute requirement of E2 (Del Vecchio et al., 1992, DiMaio and Settleman, 1988, Groff and Lancaster, 1986). The role of E2 in replication is not entirely clear. E2 has been found to alleviate nucleosomal-mediated repression of replication by altering the chromatin structure around the ori, thus allowing accessibility of cellular replication factors (Li and Botchan, 1994). It also directs E1 to bind to the ori DNA (which it normally has only low specificity for), this cooperation leads to activation of DNA replication (Yang *et al.*, 1991). However, this function can be bypassed if E1 is expressed at high levels (Mannik et al., 2002). Therefore, it appears that E2 plays more of an auxiliary or facilitory role in DNA replication.

In this study, we report the autonomous replication of HPV16 containing plasmids in the absence of E1 and E2 ORFs or their protein products. The E1 and E2 ORF mutants were able to replicate at a level comparable to wildtype. Similar results were obtained from transient replication assays using HPV16 genome that contains a deletion of the LCR and progressive deletions extending from the LCR to E2 coding region. The level of viral trans-factor-independent replication, is clearly above background, thus, we conclude that it represents an intrinsic activity of the HPV genome, separate from the E1-dependent ori. Since E1 is a helicase that plays a role in replication by recruiting DNA polymerase $\alpha$ and unwinding DNA, we infer that such functions must be substituted by cellular helicases, such as mini-chromosome-maintenance proteins (MCMs), and Werner and Bloom helicases.

E1-E2 independent replication may contribute to a true form of latency, in which the virus minimizes its expression of viral proteins in host cells and keeps the viral copy number low by utilizing cellular replication proteins under tight S phase control. A mechanism of DNA replication that is controlled by the cell has been well studied in EBV, whose DNA is replicated one time per S phase in latently infected cells (Lindner and Sugden, 2007, Yates and Guan, 1991). This mode of replication is achieved through replication licensing proteins, MCMs an ORC, which are restricted to late mitosis and G1 phase of cell cycle (Maiorano *et al.*, 2000). These proteins assemble on the latent origin of replication, OriP, of EBV and are also known to complex with the latent origin of replication of KSHV DNA (Stedman *et al.*, 2004). The low level of viral genome replication, as regulated by cellular factors, is a beneficial and common strategy for episomal viruses to establish latency. We suggest that HPV may adopt this mechanism to control its genome replication in favor of DNA maintenance during its persistence phase in the host. Based on the similar magnitude of wildtype versus E1/E2 ORF mutants, our results suggest that E1-E2-independent replication may be

the preferred mode of replication upon the initial infection and that higher levels of E1 alter the mode of replication later in the infectious process.

To date, it is not clear whether the cellular DNA replication licensing proteins are associated or necessary for replication in the absence of E1 and E2 with HPV16 DNA. It is interesting that E1, which is an ATP-dependent helicase and origin-binding of HPV has many similarities to cellular MCM proteins. In fact, recent preliminary work has shown that E1 directly interacts with MCM2 and MCM6 of the MCM hexamer (Kenneth Alexander, personal communication). Since this binding is specific, it is likely that E1 and MCMs share conserved structures that are interchangeable to form a hybrid-helicase hexamer at origin of replication. This possibility is currently under investigation. Our studies suggest that E1-E2 independent replication may act to preserve a latent-like state where MCMs are substituted for E1 and replication is carried out by cellular proteins that control the replication in once-per-cell cycle manner (Hoffmann et al., 2006). This strict replication is overcome by expression of E1 that switches replication to a multiple DNA copy per-cell-cycle mode, perhaps by directly modifying MCM complexes.

Under conditions in which E1 and E2 were not expressed exogenously, WT HPV16 and all mutants, exhibited low-copy replication efficiency in the human cervical cell C33A and human embryonic kidney cell 293. This result is in contrast to experiments in which ectopic expression of E1 and E2 was provided, in which robust a wide range of mammalian cells were reported to support LCR-dependent replication (Chiang et al., 1992, Del Vecchio et al., 1992, Kuo et al., 1994). Cell and species specificity in viral replication have been reported in the other members of the papovavirus family; SV40 and polyomavirus replication specificities rely on species-specific interactions between large tumor antigen and the cellular DNA polymerase $\alpha$/primase complex and the single-stranded DNA binding protein (Murakami *et al.*, 1986, Schneider *et al.*, 1994, Smith *et al.*,

2002). It is likely that the replication restriction also exists for papillomaviruses. As results demonstrated in this and previous studies (Farwell *et al.*, 2000, Togawa and Rustgi, 1995), variation of cell and tissue restriction for HPV replication suggests that HPVs rely to a greater degree on specific cellular factors than previously thought. This may explain the strict host range of these viruses that is, in part, implicated by the presence of cellular factors necessary for the initiation of DNA replication.

Our study has demonstrated that E1-E2-independent HPV DNA replication occurs by alternate origin activity outside of the LCR. The replication efficiency is relatively low and cell-type restricted, which makes sense for a virus which spends much of its effort to avoid immune surveillance. It is speculated that cellular factors may play a dominant role in this mode of replication. Potential mechanisms that may account for E1-E2-independent replication and maintenance may include recruitment of transcription factors and/or recruiting of cellular replication mechinary by cellular proteins. Our recent results suggest that cellular chromosomal segregation (centromeric, metaphase chromatin, and telomeric) regulation proteins are reasonable candidates for this role. Further dissection of the cis-and trans-acting components that regulate E1-E2-independent HPV replication is required to understand its role in the context of the viral life cycle.

Figure 2.1 Transient replication of HPV16 DNA in different cell types. Five µg of plasmid containing HPV16 genomic DNA were transfected into various cell lines; baby hamster kidney cell (BHK), American green monkey kidney cell (Vero), human osteosarcoma cell (U2OS), human cervical carcinoma cell (C-33A) and human embryonic kidney cell (293). After 4 days of transfection, low molecular weight DNA was extracted from 1 x $10^7$ cells by Hirt method. During extraction, 10 ng of pUC19 DNA was added as a "spiked plasmid" to moniter the recovery of the plasmid DNA from transfected cells and to test the completeness of DpnI digestion. For the Southern analysis, 10% of Hirt-DNA was digested with HindIII to linearize the plasmid and 90% was digested with both HindIII and DpnI. The blot was probed with random-primed $^{32}$-P labeled pUC19 DNA. As a control, linearlized plasmid containing HPV16 at concentrations of 100 and 10 pg was loaded on the left of the blot.

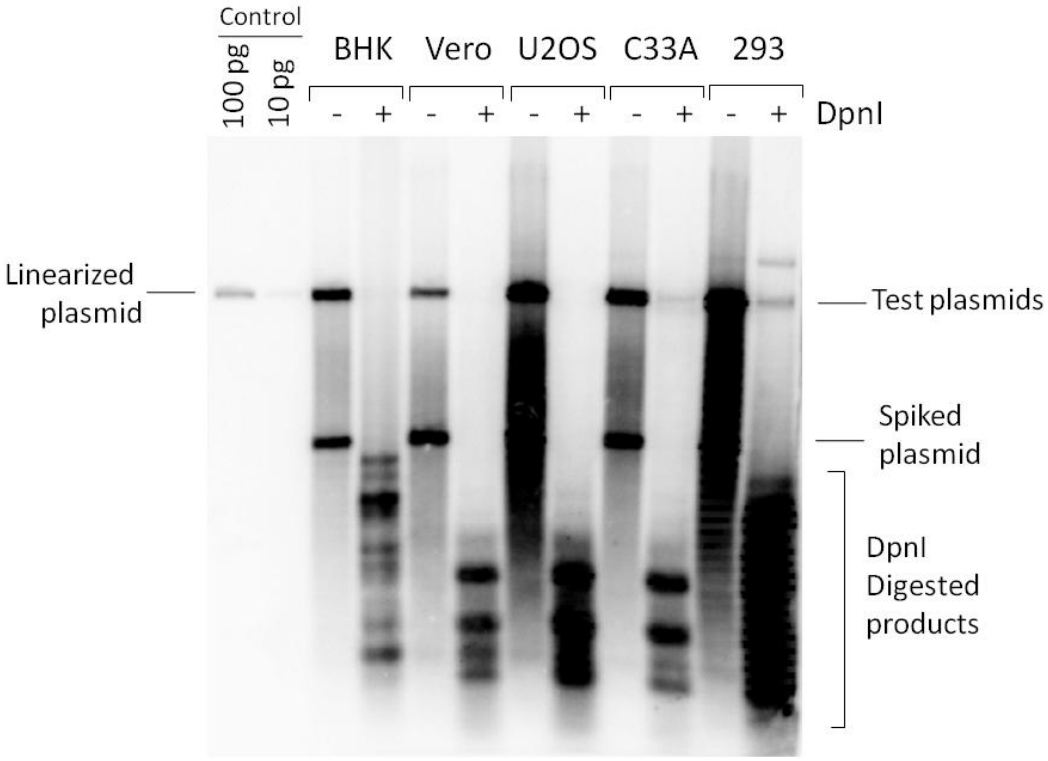
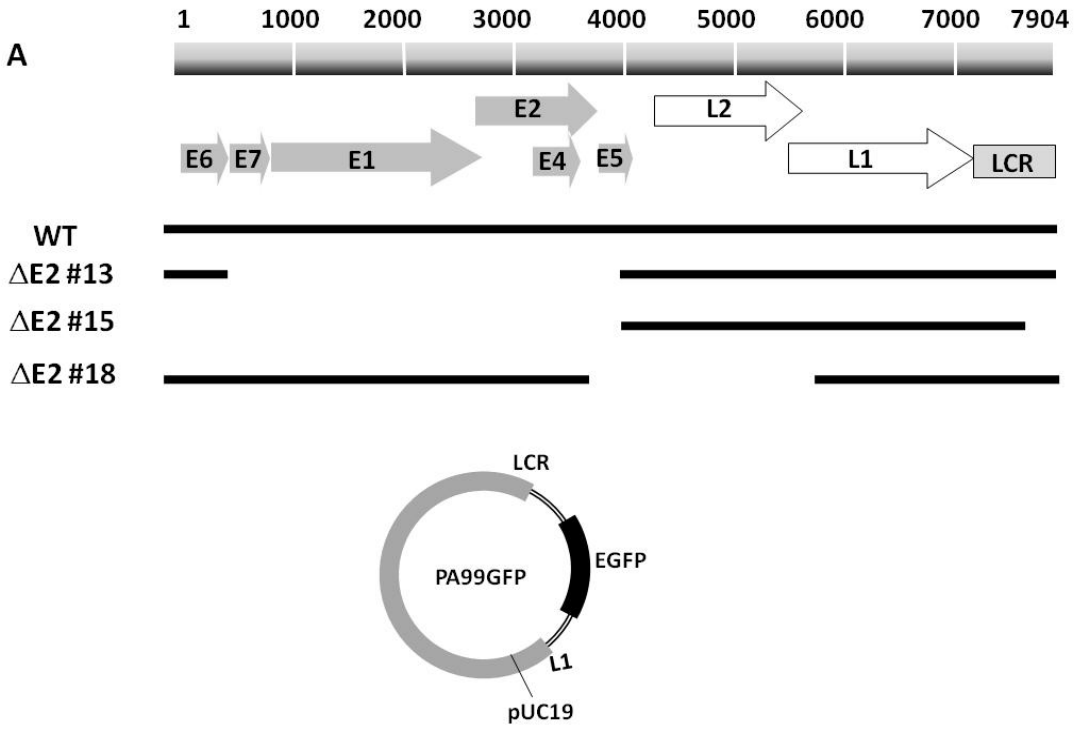

Control
100 pg
10 pg
BHK
Vero
U2OS
C33A
293
- + - + - + - + - +
DpnI
Linearized plasmid
Test plasmids
Spiked plasmid
DpnI Digested products

Figure 2.2 Replication of HPV16 DNA independently of E1 and E2 proteins. (A) Maps of PA99GFP and its mutant derivatives. The top bar represents a linear map of the HPV16 genome with responsive eight ORFs shown in arrows and the LCR indicated by a gray bar. The lines below show HPV16 segments, designated wild type (WT) and respective deletion mutants. PA99GFP contains an entire HPV16 DNA, an enhanced green fluorescent protein (EGFP) under a CMV promoter in a pUC19 backbone. The mutants ΔE2-#13, ΔE2#15, and ΔE2#18 harboring HPV16 DNA sequenced denoted by solid bars were created from pPA99GFP as described in Materials and Methods. Autoradiograms of representative Southern blots show extrachromosomal DNA (HindIII-digested products) and replicated DNA (DpnI-resistant) from transient replication assays with WT HPV16 and mutants in C33A (B) or 293 (C). Cells were transfected with wild type or mutant HPV16 DNA containing plasmids as indicated. At 4 days post-transfection, extrachromosamal DNA were isolated from 1 x $10^7$ cells. For Southern analysis, 10% of each sample was linearized with single cutting enzyme, HindIII and 90% was digested with HindIII and DpnI. Blots were hybridized with random-primed radiolabeled pUC19 DNA. The blots with HindIII digested DNA were exposed for 2 h whereas the ones with double digestion (HindIII + DpnI) were exposed for 4 days. The pUC19 backbone plasmid was used as the “spiked plasmid” to monitor the efficiency of plasmid recovery and completeness of DpnI digestion. The control DNA shown on the left, contains 1 ng, 100 pg, and 10 pg of linearized PA99GFP.


A
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3000
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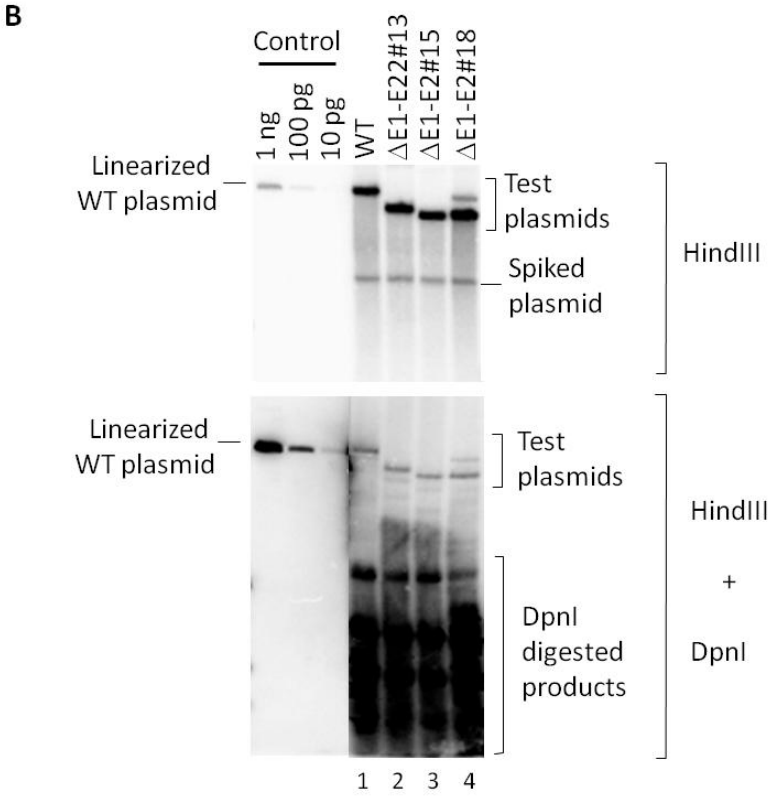
6000
7000
7904
E2
L2
E6
E7
E1
E4
E5
L1
LCR
WT
ΔE2 #13
ΔE2 #15
ΔE2 #18
LCR
EGFP
PA99GFP
L1
pUC19


B
Control
1 ng
100 pg
10 pg
WT
ΔE1-E22#13
ΔE1-E2#15
ΔE1-E2#18
Linearized WT plasmid
Test plasmids
Spiked plasmid
HindIII
Linearized WT plasmid
Test plasmids
DpnI digested products
HindIII + DpnI
1 2 3 4

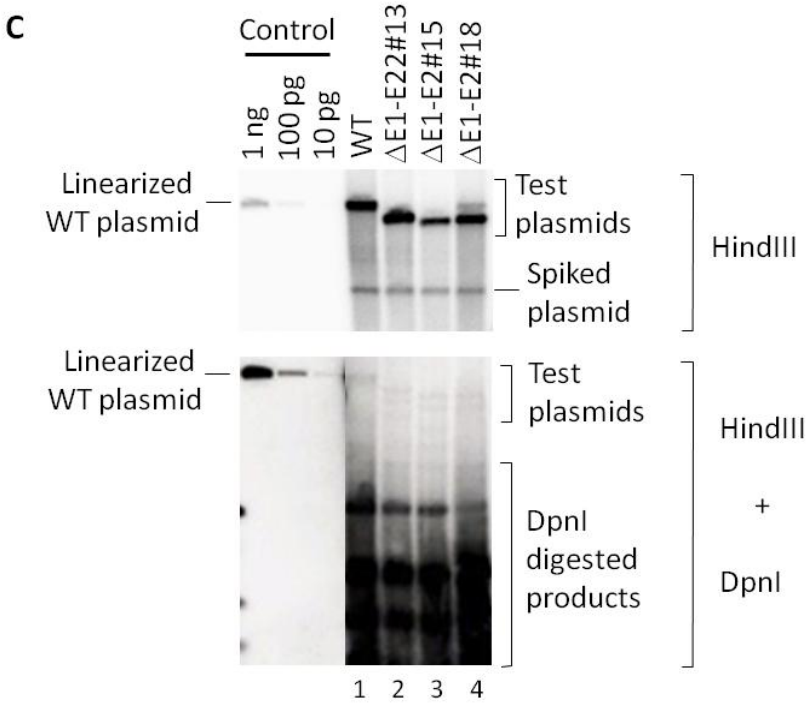
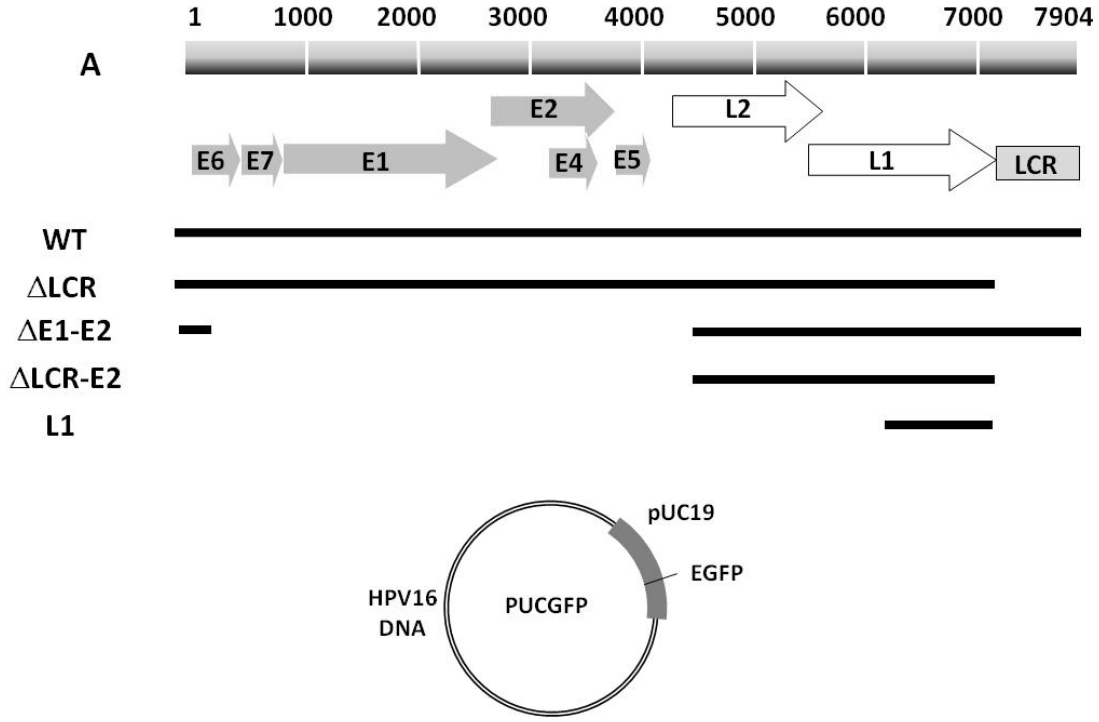
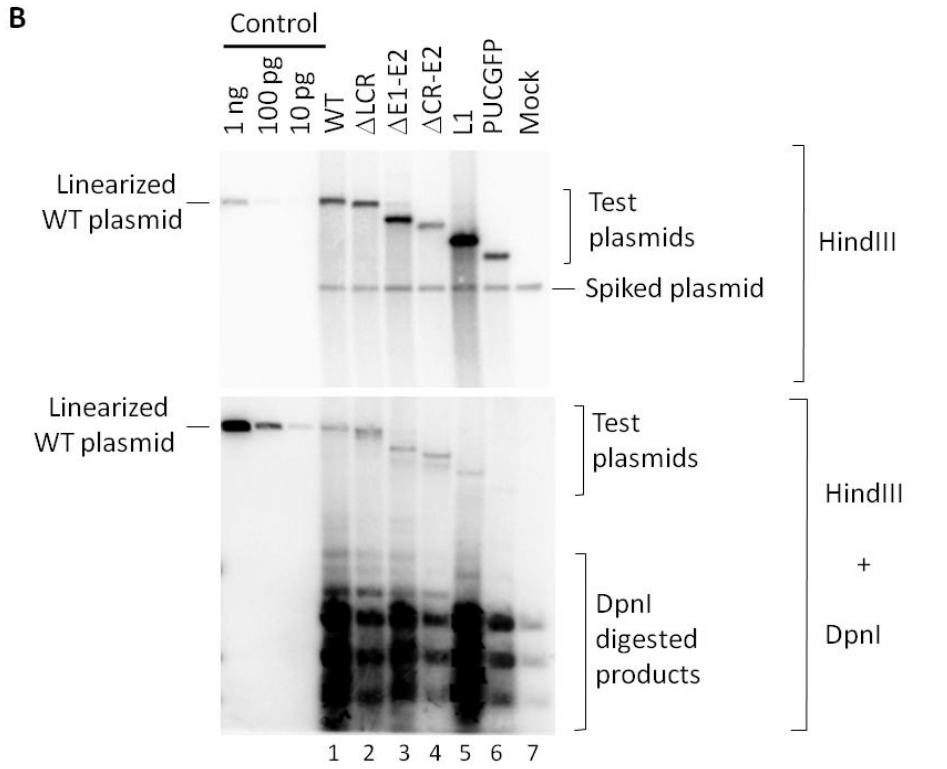
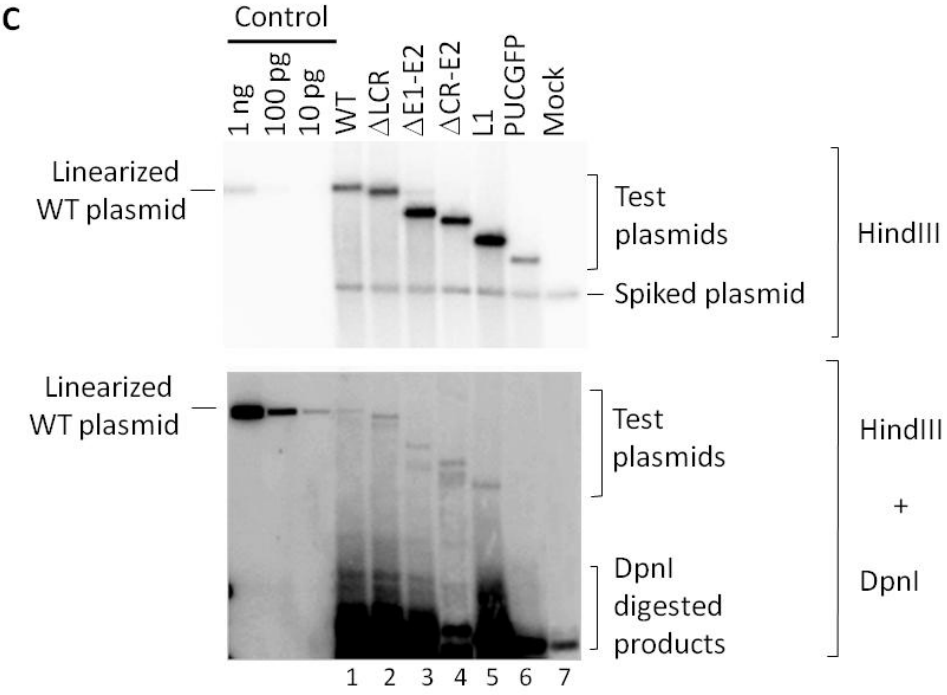

C
Control
1 ng
100 pg
10 pg
WT
ΔE1-E22#13
ΔE1-E2#15
ΔE1-E2#18
Linearized WT plasmid
Test plasmids
Spiked plasmid
HindIII
Linearized WT plasmid
Test plasmids
DpnI digested products
HindIII + DpnI
1 2 3 4

Figure 2.3 Transient replication of HPV16 mutants with LCR and E1-E2 deletions. (A) Schematic representation of PUCGFP comprising an EGFP in a pUC19 backbone. An entire sequence and partial fragments of HPV16 DNA shown in solid lines were inserted into PUCGFP at BamHI to generate WT and mutants designated ΔLCR, ΔE1-E2, ΔLCR-E2, and L1 as described in Meterials and Methods. Replication efficiencies of the HPV16 WT and mutants are evaluated by a transient transfection assay. Autoradiograms of representative Southern blots show replicated DNAs from transient transfection assays in C33A (B) and 293 (B). The assay was performed as described in Fig 2.2 legend. Ten pg to 1 ng of linear plasmid containing the entire HPV16 DNA, loaded on the left, were used as controls.


1
1000
2000
3000
4000
5000
6000
7000
7904
A
E2
L2
E6
E7
E1
E4
E5
L1
LCR
WT
ΔLCR
ΔE1-E2
ΔLCR-E2
L1
pUC19
EGFP
HPV16
DNA
PUCGFP


B
Control
1 ng
100 pg
10 pg
WT
ΔLCR
ΔE1-E2
ΔCR-E2
L1
PUCGFP
Mock
Linearized WT plasmid
Test plasmids
Spiked plasmid
HindIII
Linearized WT plasmid
Test plasmids
DpnI digested products
HindIII + DpnI
1 2 3 4 5 6 7


C
Control
1 ng
100 pg
10 pg
WT
ΔLCR
ΔE1-E2
ΔCR-E2
L1
PUCGFP
Mock
Linearized WT plasmid
Test plasmids
Spiked plasmid
HindIII
Linearized WT plasmid
Test plasmids
DpnI digested products
HindIII + DpnI
1 2 3 4 5 6 7

# CHAPTER 3

# Analysis of *cis*-elements that Facilitate Extrachromosomal Persistence of Human Papillomavirus Genomes

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**ABSTRACT**

Human papillomaviruses (HPVs) are maintained latently in dividing epithelial cells as nuclear plasmids. Two virally encoded proteins, E1, a helicase, and E2, a transcription factor, are important players in replication and stable plasmid maintenance in host cells. Recent experiments in yeast have demonstrated that viral genomes retain replication and maintenance function independently of E1 and E2. Flow cytometry studies of EGFP-reporter vectors containing subgenomic HPV fragments with or without a human ARS (hARS), revealed that six fragments located in E6-E7, E1–E2, L1, and L2 regions showed a capacity for plasmid stabilization in the absence of E1 and E2 proteins. Interestingly, four fragments within E7, the 3' end of L2, and the 5' end of L1 exhibited stability in plasmids that lacked an hARS, indicating that they possess both replication and maintenance functions. Two fragments lying in E1–E2 and the 3' region of L1 were stable only in the presence of hARS, that they contained only maintenance function. Mutational analyses of HPV16-GFP reporter constructs provided evidence that genomes lacking E1 and E2 could replicate to an extent similar to wild type HPV16. Together these results support the concept that cellular factors influence HPV replication and maintenance, independently, and perhaps in conjunction with E1 and E2, suggesting a role in the persistent phase of the viral lifecycle.

**INTRODUCTION**

Human papillomaviruses (HPVs) are small circular double-stranded DNA viruses that establish long-term persistent infections in squamous epithelial cells. The viral life cycle is tightly linked with the differentiation state of the host cells. It is thought that an early amplificational event occurs following virus entry into basal-layer cells. The viral DNA persists as a nuclear episome in infected cells. In the non productive stage of infection, HPVs replicate at low copy number in mitotically active basal-layer cells within stratified epithelia (Howley, 1996). In the suprabasal keratinocyte layers, the viral genome undergoes amplification, which yields from 100 to 1000 viral copies per cells. The percentage of differentiated cells that amplify viral DNA is often very low (Flores et al., 2000). Papillomaviruses utilize two viral factors, E1, a helicase, and E2, a transcriptional activator and auxiliary replication factor (Rabson *et al.*, 1986, Ustav and Stenlund, 1991, Ustav et al., 1991). The E1 protein forms hexamers and unwinds viral DNA similarly to cellular helicases, such as Werner's (WRN) and Bloom's (BLM) syndrome helicases and minichromosome maintenance (MCM) proteins (Ellis *et al.*, 1995, Fouts *et al.*, 1999, Kass *et al.*, 1997, Mohaghegh *et al.*, 2001, Patel and Picha, 2000). E1 has homology to SV40 DNA helicase, and Tag, and both proteins direct pol$\alpha$ activity to their respective origin (Clertant and Seif, 1984, Mansky *et al.*, 1997, Park *et al.*, 1994). In the final phase of HPV infection, the virus amplifies differentiating suprabasal cells, and finally viral capsid are produced (Howely, 1996).

HPV genomes can persist in proliferating keratinocytes for years or decades. This persistent phase of the viral lifecycle is characterized by detectable levels of viral genome but the absence of virus production (Stubenrauch and Laimins, 1999) is likely a strategy to evade immune surveillance. Maintenance of viral episomes requires that newly synthesized DNAs be faithfully partitioned to daughter cells during mitosis. A similar strategy is employed by Epstein-

Barr virus (EBV) and Koposi's sarcoma-associated herpesvirus (KSHV). These viruses maintain their genomes in dividing cells through the function of a virally encoded anchoring protein, which links the viral DNAs, via repeat binding sites, to host mitotic chromosomes (Ballestas *et al.*, 1999, Ballestas and Kaye, 2001, Harrison *et al.*, 1994, Lupton and Levine, 1985, Sears *et al.*, 2004, Yates et al., 1984, Yates *et al.*, 1985). The same scenario has also been observed in bovine papillomavirus type 1 (BPV1). Several studies in BPV1 have shown that the major players, E1 and E2, are key players in replication along with multiple E2 binding sites located in the long control region (LCR) (Abroi *et al.*, 2004, Bastien and McBride, 2000, Ilves et al., 1999, Piirsoo et al., 1996, Ustav et al., 1991, Wu *et al.*, 2000). However, some studies suggest that BPV1 E1 contributes to viral establishment but is not required for the maintenance stage of viral life cycle (Kim and Lambert, 2002).

The E2 viral protein contributes to genome persistence by linking viral DNAs, through specific DNA binding sites ($ACCGN_4CGGT$), to mitotic chromosomal proteins via an interaction with the E2 N-terminus (Abroi et al., 2004, Ilves et al., 1999, McBride et al., 2006). This mechanism was first observed in BPV1 studies, in which E2 was found to stably bind to mitotic chromosomes via a cellular protein, bromodomain protein 4 (Brd4) (Baxter and McBride, 2005, Baxter et al., 2005, You et al., 2004). However, recent observations of mitotic chromosome interactions with E2 protein from multiple papillomaviruses have revealed that there is significant variation in the timing of binding and the specificity to the binding partner, Brd4 (McBride et al., 2006). The E2 proteins from $\alpha$-papillomaviruses (HPV11, HPV16, HPV31, and HPV57) are able to bind to mitotic chromosomes but only in telophase of mitotic process (Oliveira *et al.*, 2006). Further studies have suggested that the chromosome attachment of HPV E2s is independent of Brd4 since the Brd4-binding affinities of the E2s are much lower than that

of BPV1. Additionally, point mutations in E2 that eliminate the Brd4 binding do not affect the mitotic chromosomal localization of these proteins (McPhillips et al., 2006). This indicates that HPVs, particularly in the alpha genus, have evolved a different strategy to maintain and equally distribute their genomes to daughter cells.

In the case of BPV1, a total of 17 E2 binding site (E2BS) has been identified; 12 of them are located within the LCR region (Li et al., 1998). For efficient partitioning and long-term persistence of episomal BPV DNA, and element containing at least 10 E2BSs, known as the minichromosome maintenance element (MME) was needed (Piirsoo et al., 1996). This minimal number of E2 binding sites sufficient to provide the minichromosome maintenance function exceeds the number of sites generally found in LCR of many HPV types. Taken together, E2 and its binding sites, may provide maintenance/partitioning function in HPVs with the accompaniment of cellular maintenance factors. Thus, we hypothesize that stable maintenance of the HPV genomes in proliferating cells requires additional cellular factors for efficient retention and faithful segregation of their genomes.

In previous experiments in *Saccharomyces cerevisiae*, we demonstrated that HPV16 genomes can be replicated and stably maintained in the absence of any particular viral protein, notably, E1 and E2 (Angeletti et al., 2002, Kim et al., 2005). The existence of an E1 and E2-independent mode of replication and maintenance of HPV is supported by mapping studies in yeast (Kim et al., 2005, Kim and Lambert, 2002). In those studies, elements, referred to as *mtc* elements, present outside LCR sequence that could substitute for autonomously replicating sequence (ARS) and centromere (CEN) function in yeast. The results suggest that remaining *cis*-elements are recognized by cellular factors and confer both replication and maintenance functions to the viral genome.

In the present study, we confirm and E1- and E2-independent mode of viral replication in human cells by using a series of HPV16 deletion mutants. Those mutants that lack E1, E2, and LCR sequences showed a comparable level of replication to the wild type genome in short-term replication assays. This experiment provided firm evidence that E1, E2, and E2BSs were not entirely essential for viral replication and that the requirement for viral factors could be supplanted by cellular replication machinery, under certain conditions. We hypothesized that undefined *cis*-elements outside the LCR, and recognized by cellular factors, could confer a degree of maintenance activity. In order to determine which regions of the genome had intrinsic maintenance function, we performed a complementation assay in mammalian cells using plasmids containing HPV16 subgenomic fragments, an EGFP cassette, with or without a previously defined human ARS (hARS) (Masukata *et al.*, 1993). We then tested each plasmid for short and long-term extrachromosomal replication and we measured their mitotic stability. The results described here support the concept that HPVs make use of E1 and E2-independent *cis*-acting replication and maintenance signals, which likely allows greater regulation on copy number and segregation.

## MATERIALS AND METHODS

### *Mammalian cells and transfection methods*

Human embryonic kidney (HEK) 293, Hela and HaCaT were grown in Dulbecco's modified Eagle's medium supplemented swith 10% fetal bovine serum in a 5% $CO_2$ incubator at 37°C. All transfections were performed using Dreamfect reagent (Ozbiosciences), 2 μg of DNA and 4 x $10^5$ cells in 6-cm plates. Cells were seeded in plates for 16 to 24 h prior to transfection.

***Plasmids and library constructions***

To identify *cis*-elements in HPV16 that are responsible for stability of viral genomes, we first created a vector that carries a hARS and an enhanced green fluorescent protein (EGFP) cassette. The pGFP vector (pGAB1) was fused to a 4980-Bp segment of hARS (from clone P1W1) (Masukata et al., 1993) resulting in a $hARS^{+}$ pGFP vector (PGAB1) which is able to replicate autonomously in human cells. HPV16 library constructs were then made by cloning HPV16 subgenomic fragments into pGAB1 vector (Fig. 3.3A). The HPV16 genome was digested with Sau3AI into multiple fragments ranging in size from 2.6 Kb to 18 Bp which were cloned into the pGAB1 vector using linkers (top, 5'-CGAGCTAAGCTTGCAA-3'; bottom, 5'-GATCTTGCAAGCTTAGCTCG-3'; HIndIII site (underlined)). Individual plasmids containing each fragment of the HPV16 genome were isolated.

To map regions in the viral genome that conferred both replication and maintenance functions, a second set of HPV16 library was constructed by removing the hARS from pGAB1 library plasmids by EcoRI.

To rule out any bias effects due to the SV40 promoter that exists in the previous sets of HPV16 library, the third HPV16 library was created in pUC19 carrying an expression cassette of EGFP gene. To generate and $EGFP^{+}$ pUC19 vector, an entire EGFP gene including CMV promoter was amplified from pEGFP-C1 (clontech) using 5'CMV (5'-GCATCCTCGAGGTAATCAATTACGGG-3') and 3'GFP (5'-CGAAGCTTGAGCTCGAGATCTGAG-3') primer containing XhoI site (underlined). The PCR products were trimmed with XhoI and directly inserted into pUC19 vector at the XhoI site. The resultant vector was named pUCGFP. Then, the full-length HPV16 genome that was excised from pEF399 with BamHI digest was ligated into pUCGFP at the BamHI site, resulting in

the plasmid referred to as HPV16GFP. All mutant derived from HPV16GFP, shown in Fig. 3.6, were made by multiple restriction enzyme digestion to remove certain regions of HPV16 genome and the remaining DNA was religated with T4 ligase. The ΔMSC mutant was generated by deleting the fragment between 2 MscI sites of HPV16GFP that contains entire sequence of L2 encoding region and part of the E2 gene 5' region. ΔE1-E2 was generated by deleting the StuI-PmlI fragment of HPV16GFP that contains E1 and E2 coding regions. ΔLCR-E2 was generated by deleting the StuI-PmlI fragment of HPV16GFP that contains the E2 coding region through LCR. ΔL1 was generated by deleting the PmlI-XbaI fragment of HPV16GFP that containing 5' end of L1 coding region. The last mutant, L1, was created y deleting the SmaI-PmlI fragment of HPV16GFP and left fragment in it.

***Plasmid maintenance assay***

The plasmid maintenance experiment was modified from the protocol previously described by (Vogel *et al.*, 1998). HPV16 library plasmids were transfected into human cell lines using Dreamfect. The media was changed 24 h after transfection to remove excess DNAs and residual transfection reagent. On the next day, the cells were treated with 0.5 mg/mg G418 to favor the growth of transfected cells containing the plasmids that confer G418 resistance. The EGFP-expressing cells were left under selective conditions for 4 days to allow untransfected cells to die. The surviving cells were released into nonselective media for 10–14 days and periodically subjected to analysis by flow cytometry for GFP and Southern blot analysis. In order to determine the stability of the plasmids, the relative percentages of EGFP positive cells were calculated and plotted versus the period of time after cells were released from the drug. To obtain the relative percentages of EGFP positive cells, the final percentages of EGFP positive cells, at several time points of sampling, were divided by the initial percentage of EGFP positive

cells at day 0 which is the first day cells were released from selection media. We calculated the loss rate of GFPpositive cells per cell generation and normalized with that of the most stable plasmid to obtain the loss rates per cell generation.

***Assay for plasmid DNA replication***

Cells were plated in 100 mm dishes and transfected with 5 μg of DNAs, while the cells were 50 to 70% confluent. The cells were incubated with the DNAs for 24 h, then the media was changed. For the short-term replication assay, plasmid DNAwas isolated using the Hirt method (Hirt, 1967)at 4 days after transfection. For the long-term replication assay, the transfected cells were grown in media containing 0.5 mg/ml G418 for 4 days after transfection then released to media without the drug for 10–14 days. DNA was isolated using the Hirt method. The plasmids were double-digested for 16–24 h with DpnI and XhoI for HPV16 library in pUCGFP, or with DpnI and XhoI for HPV16 library in pGAB1 or pGFP. DNA samples were electrophoresed in a 0.8% agarose gel then analyzed by Southern blot using EGFP as a probe.

***Analysis of the HPV16 genome for maintenance protein binding sites***

TheHPV16 genome was analyzed for perfectmatches with the high-mobility-group (HMG) protein consensus binding site (WWWWWWS) using the Fuzznuc program (http://bioweb.pasteur.fr/seqanal/interfaces/fuzznuc.html). The frequency of predicted HMG sites was plotted against the HPV16 genome with the threshold setting of N10 sites per 100 nucleotides. Similarly, topoisomerase II (Topo II) consensus sites (RNYNNCNNGYNGKTNYNY) were plotted with a threshold frequency of N7 sites per 100 nucleotides. The presence of single binding sites of centromere binding protein B (CENP-B; YTTCGTTGGAARCGGGA) and telomere-repeat factor (TRF; TTAGGTTA) was plotted against the HPV16 genome. Matrix or scaffolding attachment regions (MARS/SARS) were located in the HPV16 genome using the Marscan

program (http://bioweb.pasteur.fr/seqanal/interfaces/marscan.html).MARS aremade up of bipartite sequence elements of 8 Bp (AATAAYAA) and 16 Bp (AWWRTAANNWWGNNNC) within a 200 Bp distance from each other. The can be on either strand of the DNA and can overlap (van Drunen *et al.*, 1999). In addition, we included previously reported MAR locations mapped in HPV16 (Tan et al., 1998).

**RESULTS**

***E1 and E2-independent replication of HPV16***

Recent studies in yeast showed that, under certain conditions, HPV16 could replicate in the absence of functional E1 and E2 proteins (Kim et al., 2005). In order to confirm the E1 and E2-independent mode of replication of HPV16 in human cells, we generated a series of HPV16 deletion mutants cloned into a modified pUC19 vector, referred to pUCGFP that carries an expression cassette of EGFP. A panel of HPV16 deletion constructs was transiently transfected in HEK 293 cells in

parallel with a parental wild type HPV16 and vector alone. Low molecular weight DNAs were isolated by the Hirt method at 4 days after transfection and then subjected to overnight digestion with DpnI and HindIII prior to Southern blot analysis using pUCGFP as a probe. By these means, the input DNA originated from bacteria that is sensitive to DpnI was completely degraded. Only newly synthesized, DpnI-resistant, plasmids were still intact and observed as linear DNAs on the Southern blot when they were cleaved by HindIII (Fig. 3.1A). In this experiment, a similar level of replication was observed in most mutants compared to the wild type HPV16, even though they lacked E1, E2 coding regions and the LCR sequence. This result is in agreement with the data from the previous studies in yeast that give us the evidence of E1- and E2-independent mode of replication. The same experiments were performed in HaCaT and

HeLa cells and similar results were achieved, but since transfection efficiency was superior in 293 cells we chose to do the analyses in 293 cells. Many other studies have used 293 cells to identify replication and maintenance elements in EBV and KSHV (Skalsky et al., 2007, Yates *et al.*, 2000).

***Plasmid stability of HPV16 deletion mutants that lack E1 and E2 coding regions***

In order to assess the capability of wild type HPV16 and its mutant derivatives to be stably maintained in mammalian cells, the plasmids were individually transfected into 293 cells along with pCNDA3.1 (+) that confers G418 resistance. The drug-resistance property of the transfected cells allows them to outgrow untransfected cells under selective conditions. After transfection, the cells were grown under selective conditions for 4 days to enrich cells containing the plasmids and to allow plasmids to become established. After removal of selection, the stability of plasmids was evaluated by monitoring the remaining GFP positive cells in the total cell population over the course of at least 2 weeks. Using flow cytometry, we could measure percent of GFP-positive cells at different time points over several cell generations and the percent loss of GFP-positive cells per cell generation was calculated (Fig.3. 1B). The change of GFP-positive cells over time, which indicates the stability of plasmid, depends on two main processes; replication and partitioning. If both replication and partitioning of the GFP-containing plasmid occurs in dividing cells, the number of GFP-positive cells remains essentially unchanged. If only one mechanism takes place (either replication or partitioning), the number of GFP-positive cells should be decreasing. In the case of partitioning plasmids that lack replicative activity, the number of GFP-positive cells should remain constant over the course of only the first cell divisions. After each cell division, even if partitioning is functional, the copy number of the non-replicating plasmids will fall below a detectable level. As shown in Fig. 3.1B, the vector itself that did not contain any segments of the viral genome was lost at a rate of 4% per cell

generation. In contrast, all derivative mutants of HPV16 showed similar pattern of plasmid stability to that observed in wild type with the lower loss rate compared to the vector alone. Again, the mutants that lack E1, E2, and LCR regions were retained in the cells for 16 days. Interestingly, the vector that carries only L1 sequence was efficiently maintained in mammalian in the absence of selection for at least 16 cell generations. This suggests that the maintenance of HPV16 genome is independent of the viral proteins E1 and E2 and therefore the E2 binding sites as well as the viral origin of replication that are located within the LCR regions are not required for the retention of plasmid in the cells. It is likely that undefined *cis*-acting elements residing outside the LCR region would be recognized by cellular factors and that confers the stability of the plasmid.

***Mapping of E2-independent maintenance function***

In previous studies, it was discovered that PV genomes could be stably maintained in human cells in the absence of functional E1 and E2 proteins (Angeletti et al., 2002, Kim et al., 2005, Kim and Lambert, 2002). To identify such elements, we created a library of plasmids in which subgenomic fragments of the HPV16 genome, generated by digestion with Sau3AI, were inserted into pGAB1, a vector containing a human ARS element, a neomycin-resistant gene, and an expression cassette for EGFP under the strong constitutive CMV promoter. The vector backbone carries the human ARS element to provide the replication function in mammalian cells to ensure that loss of plasmids over the course of the experiment was not due to lack of such activity and to allow the identification of maintenance function in regions where no replication function resides. To examine whether this HPV16 library plasmids were able to replicate in human cells, we performed a short-term replication assay with DpnI treatment at 4 days after transfection. All HPV16 subgenomic containing plasmids replicated in 293 cells as efficiently as vector itself that does not harbor any segment of HPV16 DNA (Fig. 3.2A). Similar observed

replication efficiencies rule out the possibility of interference of replication function in long-term maintenance assays. Therefore, the HPV16 subgenomic fragments present in the pGAB1 vector, that provides replication function, would be considered as maintenance elements and this stability did not result from the advantage of replication. We noted that there was a steady loss of replication function after 10 days post-release from G418, yet plasmids persisted as evidenced by Southern analysis and by the presence of EGFP signal (Fig. 3.2B). The gradual loss of replication function and continued DNA persistence was witnessed with all constructs to varying extents. To ensure that the persisting non-replicating DNAs were not simply extracellular, we washed transfect cells with PBS and subcultured them every 3 days. Cells were then externally treated with 1 U of DnaseI prior to Southern analysis. In these experiments, persisting plasmids were indeed intracellular and episomal for greater than 14 days.

To track the maintenance function imparted by elements in HPV16 DNA, we conducted flow cytometry to quantify the EGFP signal over the course of 10–14 days after cells were released from the G418 selection. Thus, the loss of EGFP signal represents plasmid the loss and indicates differences in relative intrinsic stability of the plasmids in human cells. To analyze plasmid stability, we calculated the loss of GFP-positive cells per cell generation and normalized with that of the most stable plasmid to obtain the relative loss rates. As shown in Fig. 3.2C, the plasmids that carried certain parts of HPV16 genome were more stably maintained than was pGAB1. Among the HPV16 library plasmids, six of the nine Sau3AI subgenomic fragments (fragments A (nt 525–621), B (nt 622–870), C (nt 871–3,479), F (nt 4538–5071), G (nt 5072–6150), H (nt 6151–6950) were identified to support long-term maintenance as they exhibited lower relative loss rates compared to vector alone. Those fragments are located within two distinct regions in the HPV16 genome; the first region resides in the early genes lying from the 3’ portion of E6 ORF to the 5’ segment of E2 ORF (fragments A, B, and C) and the other region is in

the late genes that covers the 3' end of L2 and the entire L1 ORF (fragment F, G, and H) (Fig. 3.2C). Interestingly, these maintenance elements mapped in human cells are overlapping the regions of HPV16 that were previously identified as maintenance elements in yeast system (Fig.3. 5).

***Four fragments within maintenance elements contain both replication and maintenance functions***

The plasmid library in pGAB1 backbone that is mentioned above was used to map region of the HPV16 genome that provides plasmid stability. Although all plasmids exhibited similar replication efficiency, we cannot rule out the possibility that some of these fragments would contain replication activity since such elements have been mapped in HPV16 genome using a yeast system. In order to identify replication elements in the viral genome, we generated another plasmid library in the absence of hARS by removing the hARS element from each HPV16 library constructs that were made in pGAB1. These plasmids allow us to identify the elements that support DNA replication based on their ability to substitute for the function of hARS. We tested whether these plasmids were able to replicate in 293 cells using a short-term replication assay and found that most of them replicated to differing extents (Fig. 3.4A). The shortterm replication assay showed the strongest initially detectable levels of DpnI-resistant DNA in fragments A (nt 525–621), B (nt 622–870), C (nt 871–3479), E (nt 4361–4519), F (nt 4538–5071), G (nt 5072–6150), I (nt 7014–524) (Fig. 3.4A). However, in long-term maintenance assays, using flow cytometry, we found that plasmids harboring 4 subgenomic fragments were lost at the slower rate compared to vector alone, pEGFP, whereas those baring other fragments lost rapidly. The fragments that contained both replication and moderate to significant levels of maintenance function, included fragments A (nt 525–621), B (nt 622–870), F (nt 4538–5071), G (nt 5072–6150) (Fig.3. 4B). Interestingly, these fragments are located within two regions in the

viral genome that we identified as maintenance elements using the pGAB1 vector (Fig.3. 2B). The first region is in partial portion of E6 and E7 whereas the other one is in L2 and 5' part of L1. We noted that the vector itself (pUCGFP) had some replication activity and was maintained to an extent over 10 days, however the regions A, B, F and G were maintained to a greater degree than the vector alone.

***Analysis of the HPV16 genome for maintenance factor binding sites***

We reasoned that factors that function in cellular chromosome maintenance are most likely to contribute to HPV maintenance, either independently, or in conjunction with E2. Therefore, we mapped the HPV16 genome, for the presence of HMG, CENP-B, TRF, MAR/SAR and Topo II binding sites, which others previously showed influence EBV maintenance (Deng et al., 2002, Hung *et al.*, 2001, Masumoto *et al.*, 1989). We discovered that the HPV16 genome contains high concentrations of Topo II sites in the LCR, L1, L2 and E7 ORFs, as well as HMG sites overlapping in L1 and E1. In addition, at least eight CENP-B binding sites were detected in the early and late regions of the genome. Of particular interestare the locations of MARS in L2, overlapping flanked by TRF binding sites. MAR elements are known to provide stability to replicating episomes in human cells (Cossons *et al.*, 1997, Papapetrou *et al.*, 2006) and mapping studies have identified MARS in HPV16 in E6, E1, E5, L2, and L1-LCR regions (Tan et al., 1998).

**DISCUSSION**

To establish a persistent infection, HPVs gain access to mitotically active basal-layer keratinocytes where low-copy replication begins. Viruses that replicate their DNA extrachromosomally, such as HPVs, EBVand KSHV, possess high fidelity mechanisms to maintain their genomes. The primary threats to stability of viral genomes are loss by diffusion through the nuclear pores, loss during mitosis, or by nuclease-mediated decay, and integration into the host genome (Calos and Sclimenti, 1998, Deng et al., 2002). Viruses combat these problems by: (i)

nuclear retention and compartmentalization of genomes, thus protecting them from loss and degradation (Deng et al., 2002, Hung et al., 2001, Jankelevich *et al.*, 1992, Nielsen *et al.*, 2000, Swindle *et al.*, 1999, van Brabant *et al.*, 1999) and (ii) improving segregation efficiency by attaching genomes to mitotic machinery (Ikeno *et al.*, 1998, Kanda *et al.*, 2001, Lehman and Botchan, 1998, Longtine et al., 1992).

During the maintenance phase, it is thought that low levels of E1 and E2 proteins are expressed in the infected basal cells. Given this and the fact that HPV genomes can persist for decades, this raises the question of whether cellular factors have a more prominent role in genome maintenance of HPVs than previously expected.

BPV1 E2 has been shown to interact with a chromatinbinding bromodomain protein (Brd4), thus implicating it as the mitotic chromosome anchor point for viral genomes (McBride *et al.*, 2004, You et al., 2004). The resulting model proposed for all PVs, is that chromosome anchoring allows equal partitioning of newly synthesized DNAs to daughter cells (McBride et al., 2004). E2 from BPV and many HPVs (types 1, 4, and 8) have been shown to bind tightly to mitotic chromosomes. However, HPVs (types 11, 16, 31 and 57) appear to bind mitotic chromosomes to a much weaker extent (Oliveira et al., 2006). In specific, ⟨-HPV E2 proteins could be detected near chromosomes in prophase and telophase, but not in metaphase or anaphase (Oliveira et al., 2006). A potential explanation for this disparity could be that partitioning observed among the HPVs could be dependent to a greater extent on cellular factors than HPVs for which E2 binds mitotic chromosomes tightly. Recent studies have shown that the interaction of E2 and Brd4 is not required for genome partitioning of all papillomaviruses, given by the fact that several HPV E2 proteins are capable of associating with mitotic chromosomes in the absence of Brd4 binding. Mutations in E2 have been described that disrupt the Brd4 interaction but do not affect the association of E2 with mitotic chromosomes

(McPhillips et al., 2006). Thus, segregation of HPVs by interaction with chromosomes may occur through different cellular anchoring partners and the involvement of E2 may differ between genotypes. Ultimately, the roleof Brd4 in HPV segregation remains in question since recent work has shown that Brd4 functions as a chromatin-binding transcriptional regulator and can function as a silencer (McPhillips et al., 2006, Wu and Chiang, 2007, Wu *et al.*, 2006). Further studies have demonstrated that though Brd4 has a role in transcription of viral genes, it is not required for maintenance for all PVs.

In addition to Brd4, E2 has been shown to interact with a cellular helicase (ChlR1), which was initially discovered by an interaction with the yeast homologue of ChlR1 (Parish et al., 2006). In further studies, siRNA down-regulation of ChlR1 resulted in a lack of E2 association with mitotic chromosomes, indicating a potential role for ChlR1 in loading of E2 onto chromosomal anchoring sites. Furthermore, a mutant of BPV E2 (W130R), which does not associate with mitotic chromosomes also fails to bind ChlR1 (Parish et al., 2006). Dao and colleagues described an HPV11 E2 interaction with mitotic spindles leading to an alternative model for E2-dependent genome segregation (Dao *et al.*, 2006, Van Tine et al., 2004). Although these models disagree on the anchoring target for segregation, the role of E2 as the tethering factor is well accepted.

Motivating our studies, was the dramatic disparity in the number of E2BSs in PV genomes. For example, BPV1 has at least 17 E2BSs, of which, at least 10 E2BSs are required for stable replication, per a study by Piirsoo et al. (Piirsoo et al., 1996). In contrast, HPVs usually have 4 E2BSs, only 3 of which are required for stable replication (Stubenrauch *et al.*, 1998). In addition, certain regions of HPV16 genome have been shown to interact with nuclear matrix that might be involved in nuclear retention of the viral genome (Tan et al., 1998). These observations reinforce the concept that HPVs may rely to a lesser extent on E2 than BPV1 for

stable replication. We therefore, hypothesized that unknown *cis*-elements, together with their cellular binding partners, contribute to ⟨-HPV maintenance. In our ongoing studies of HPV replication in yeast, we have found that 3 E2BSs are insufficient to restore stability to an unstable yeast plasmid ($ARS^+$ $CEN^-$) (unpublished data). Even in the presence of E2, E2BS-containing plasmids lacking a CEN become integrated into the yeast chromosome. In contrast, the HPV genome is stably maintained in the absence of E2 in yeast, yet the addition of E2 increases the copy number and improves genome stability. This indicates that E2 requires additional cellular factors for stabilization of HPV DNA and E2BSs alone, are not sufficient.

Our studies have defined *cis*-acting elements that provide a measurable degree of stability to HPV genomes independently of E1 and E2 functions. We created vectors that had either a complementing replication element (hARS) or not, each containing Sau3AI fragments of the HPV16 genome (Figs. 3.3A–C).

The pGAB1 plasmid, mentioned above, was used to map the regions of the HPV16 genome that provides plasmid stability. Although all plasmids exhibited similar replication efficiency, we cannot rule out the possibility that some of these fragments would contain replication activity since such elements have been mapped in HPV16 genome using a yeast system. In order to identify replication elements in the viral genome, we generated another plasmid library in the absence of hARS by introducing individual Sau3AI-HPV16 subgenomic fragments in pGFP, a derivative vector of pGAB1 that lacks hARS portion (Fig. 3.3C). This library plasmid allowed us to identify the elements that support DNA replication based on their ability to substitute the function of hARS.We tested whether these plasmids were able to replicate in 293 cells using a short-term replication assay and found that most of them replicated to varying extents. In a long-term maintenance assay using flowcytometry, the results revealed that the vector itself was maintained somewhat over the course of 10 days. However, four subgenomic

fragments inHPV16 genome showed more stability with increasing of percent GFP-positive cells over time. Interestingly, these fragments are located within two regions in the viral genome that have been identified as maintenance elements. The first region is in partial portion of E6 and E7 whereas the other one is in L2 and 5' part of L1.

Our mapping studies revealed that we mapped the HPV16 genome for the presence of cis-acting functions that could complement replication and maintenance functions, normally provided by the ARS and CEN elements in yeast plasmids (Kim et al., 2005). In that study, we discovered that three regions within HPV16 (nucleotides 871 to 3480, nucleotides 4538 to 5072, and nucleotides 6151 to 6951) were capable of providing maintenance function to complement an ARS+/CEN− plasmid vector (pPA94).

In the current study, we found that in the absence of the hARS fragment, replication activity in HPV DNA segments could be detected, above the level of the vector (pGFP), in fragments A (nt 525–621), B (nt 622–870), C (nt 871–3478), E (nt 4361–4519), F (nt 4538–5071), G (nt 5072–6150), and I (nt 7014–524) (Figs. 3.4B and 3.5). Perhaps the strongest replication activity was from fragments C and E. The fact that there is low-level replication activity not driven by E1 or E2,may be consistent with an alternate mode of replication. We discovered that at least four fragments within the genome contain both replication and maintenance functions in fragments A (nt 525–621), B (nt 622–870), F (nt 4538–5071), G (nt 5072–6150) (Figs. 3.4B and 3.5). These data indicates that intrinsic low-level replication function could provide a wider range of copy control for HPV, depending on the level of quiescence required to evade cell-mediated immune responses present in the stratified epithelium. Furthermore, the assistance of cellular factors in viral DNA maintenance has already been established in other viral systems.

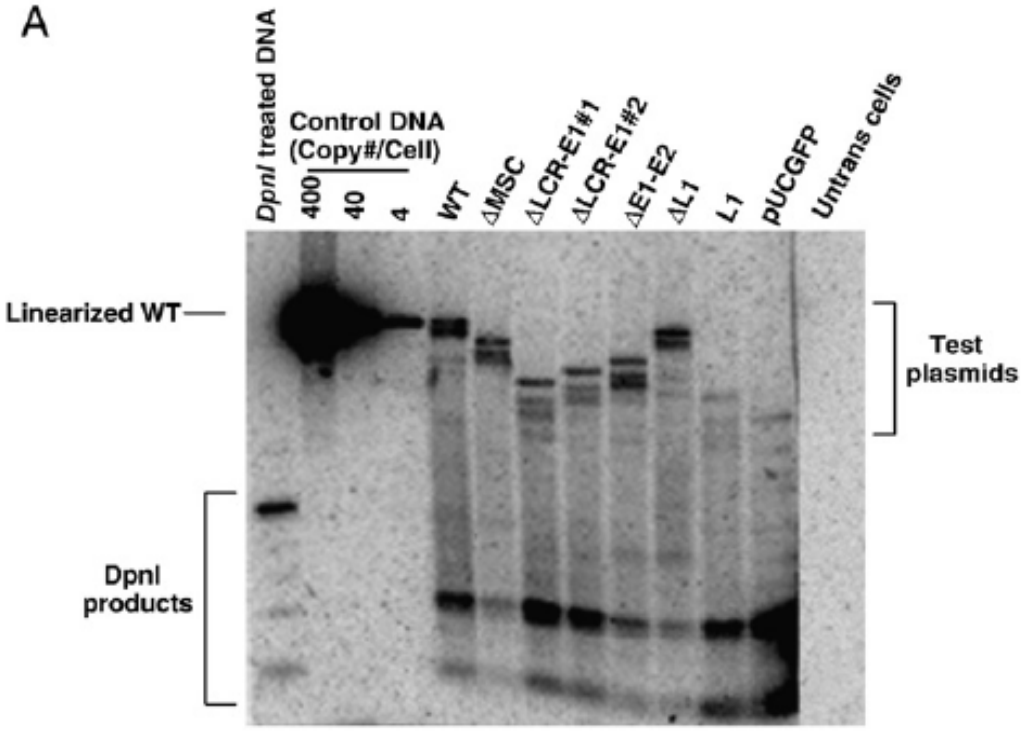
Several studies using EBV OriP, have described multiple cellular factors that influence maintenance of OriP plasmids. Examples of recently described maintenance factors which

influence EBV OriP stability are telomere-repeat binding factor 2 (TRF-2), hRap1 and poly-ADP-ribose polymerase (tankyrase) (Deng et al., 2002). It has also been shown that the histone-related proteins,HMG and H1, when fused to EBNA-1 can complement its N-terminal chromatin-binding function (Hung et al., 2001). A nucleolar protein known as EBP2, which interacts with EBNA-1, appears to be required for EBNA-1-dependent maintenance of OriP plasmids (Kapoor *et al.*, 2001). Furthermore, the cellular kinesin-like protein, Kid, which competes with EBNA-1 for its binding sites and is known to bind the metaphase plate, has also been implicated in OriP maintenance (Zhang and Nonoyama, 1994). Finally, OriP artificial chromosomes are known to be stabilized by binding CENP-B (Masumoto et al., 1989).

The concept that evolves from our studies is that cellular factors may impart a greater influence on HPV replication and maintenance than previously expected. The fact that most HPVs contain only 4 E2BSs, while BPV1 contains at least 17 E2BS, suggests HPVs have evolved in a direction, to take greater advantage of cellular factors for genome persistence. There is evidence that extrachromosomal replicating DNA viruses make use of well-conserved chromosomal maintenance proteins. For example, EBV is now known to interact with multiple chromosomal maintenance proteins (TRF, H1 histones, Kid, metaphase plate proteins, SMC, and CENP-B (Deng et al., 2002, Hung et al., 2001, Masumoto et al., 1989). The detection of multiple MARS, HMG, Topo II, TRF and CENP-B binding sites and the clustering of multiple types of binding sites in the late region in HPV16 provide some potentially important candidates to consider as cellular maintenance factors. Ultimately, these observations suggest that there is likely to be more than mechanism by which cellular factors influence HPV maintenance.

Figure 3.1 E1 and E2-independent replication and episomal maintenance of HPV16 and its mutants. (A) Short-term replication of HPV16 and deletion mutants. 293 cells were transfected with pUCGFP containing entire HPV16 genome or its mutant derivatives. Plasmids were harvested 4 days after transfection. DNA samples were double-digested overnight with DpnI and HindIII prior to Southern analysis. The left lane contains a control for the completeness of DpnI digestion of DNA that was extracted from bacteria. (B) Plasmid stability of HPV16 genome and mutants. 293 cells were cotransfected with full-length HPV16 or its mutants and pCDNA3.1 (+) that contains a neomycin-resistant gene. Cells were grown in the presence of the drug, G418, for 2 weeks. After the drug was removed, the cells were cultured under nonseletive conditions for another 16 days. Meanwhile, the

GFP-positive cells were monitored by flow cytometry at 0, 6, and 11 days after release from drug. The relative percent GFP-positive cells was calculated and plotted against time. The loss rate of each plasmid was determined as percent change of GFP-positive cell per cell generation.


A
DpnI treated DNA
Control DNA (Copy#/Cell)
400
40
4
WT
ΔMSC
ΔLCR-E1#1
ΔLCR-E1#2
ΔE1-E2
ΔL1
L1
pUCGFP
Untrans cells
Linearized WT
Test plasmids
DpnI products

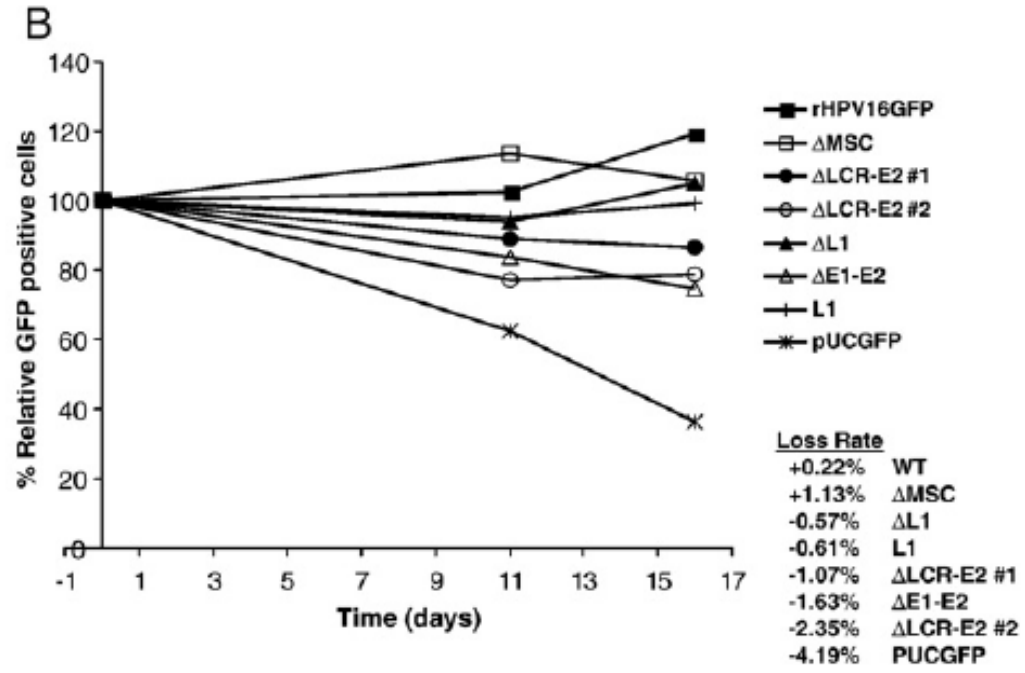
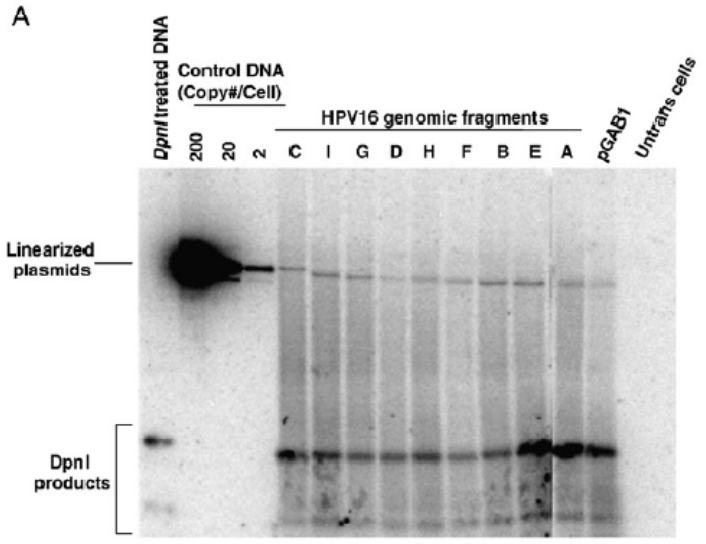
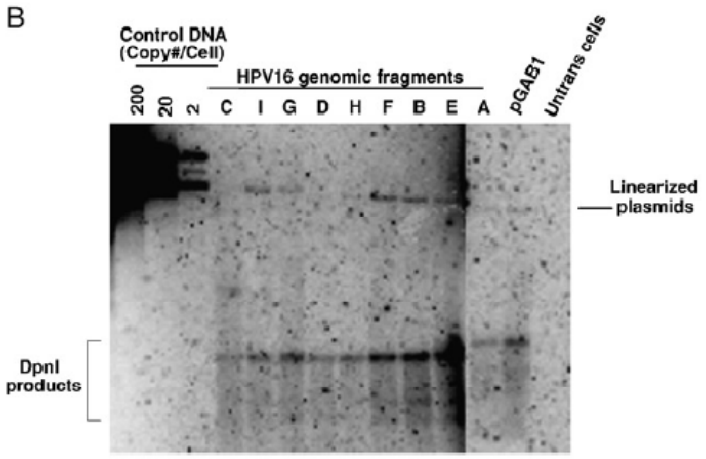

B
% Relative GFP positive cells
Time (days)
rHPV16GFP
ΔMSC
ΔLCR-E2 #1
ΔLCR-E2 #2
ΔL1
ΔE1-E2
L1
pUCGFP
Loss Rate
+0.22% WT
+1.13% ΔMSC
-0.57% ΔL1
-0.61% L1
-1.07% ΔLCR-E2 #1
-1.63% ΔE1-E2
-2.35% ΔLCR-E2 #2
-4.19% PUCGFP

Figure 3.2 Short- and long-term replication of HPV16 subgenomic DNA in the pGAB1 vector. (A) DpnI-resistant newly synthesized DNAs were linearized with XhoI and detected by Southern blot at 4 days posttransfection. XhoI-treated DNAs that indicated copy number were electrophoresed alongside. At the far left, DpnI-treated control DNA showed the completeness of DpnI cutting of DpnI-sensitive DNA from bacteria. (B) Southern analysis of Hirt-extracted DNAs at 11 days after transfection. (C) The stability of plasmids containing HPV16 subgenomic fragments was demonstrated as the relative loss rate per cell generation. The percent of GFP-positive cells was determined by flow cytometry at different time points. The change in percent GFP-positive cells per cell generation (loss rate) was computed relative to the first time point of each transfection. The loss rates of each plasmid were normalized with that of the most stable plasmid to obtain the relative loss rate per cell generation.


A
DpnI treated DNA
Control DNA
(Copy#/Cell)
HPV16 genomic fragments
200
20
2
C
I
G
D
H
F
B
E
A
pGAB1
Untrans cells
Linearized
plasmids
DpnI
products


B
Control DNA
(Copy#/Cell)
HPV16 genomic fragments
200
20
2
C
I
G
D
H
F
B
E
A
pGAB1
Untrans cells
Linearized
plasmids
DpnI
products

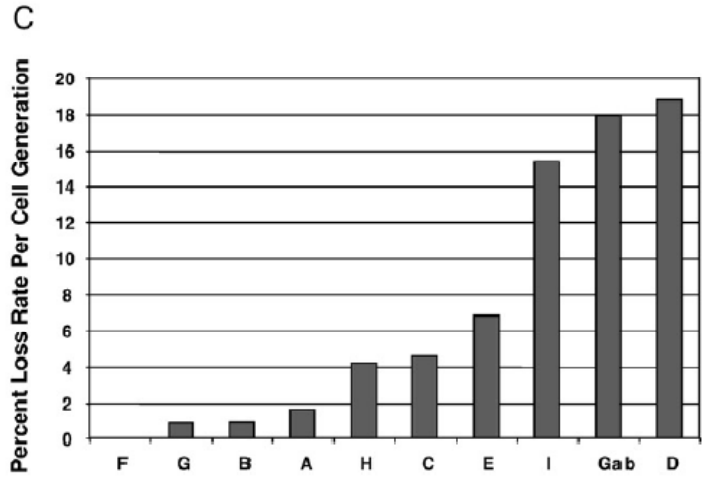

C
Percent Loss Rate Per Cell Generation
20
18
16
14
12
10
8
6
4
2
0
F
G
B
A
H
C
E
I
Gab
D

Figure 3. 3 Plasmid maps of pGAB1 and pGFP. (A) A representation of HPV16 subgenomic fragments derived fromSau3AI-treated HPV16 genome. The top bar represents a linear map of the HPV16 genome with each of eight ORFs indicated by arrows and the control region, LCR shown as a gray bar. The lines designated A to I represent the HPV16 Sau3AI subgenomic fragments (B) A map of pGAB1 used in identification of maintenance elements in HPV16 genome. pGAB1 contains an EGFP gene, a neomycin-resistance gene and an hARS element obtained from clone P1W1 (Masukata et al., 1993). To obtain HPV16 library constructs in the pGAB1 backbone, HPV16 subgenomic fragments were cloned into the HindIII site of pGAB1. (C) The map of pGFP used in identification of replication elements within theHPV16 genome. pGFP is an hARS-negative version of pGAB1. HPV16 library constructs in pGFP backbone derived from removal of the hARS portion from pGAB1 plasmids.

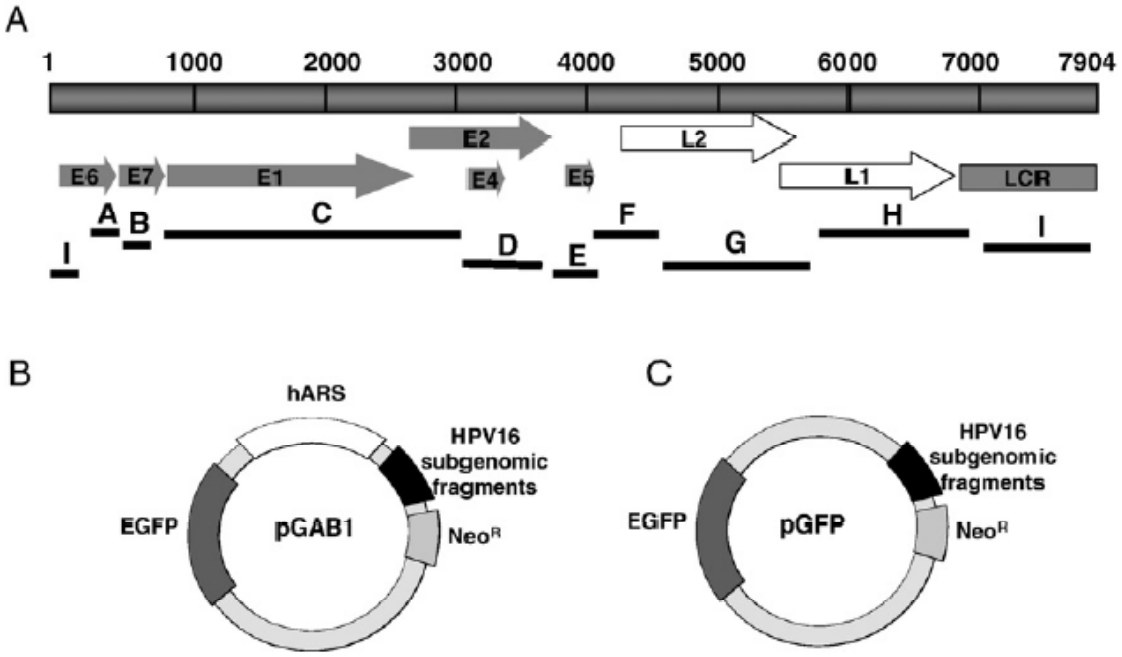
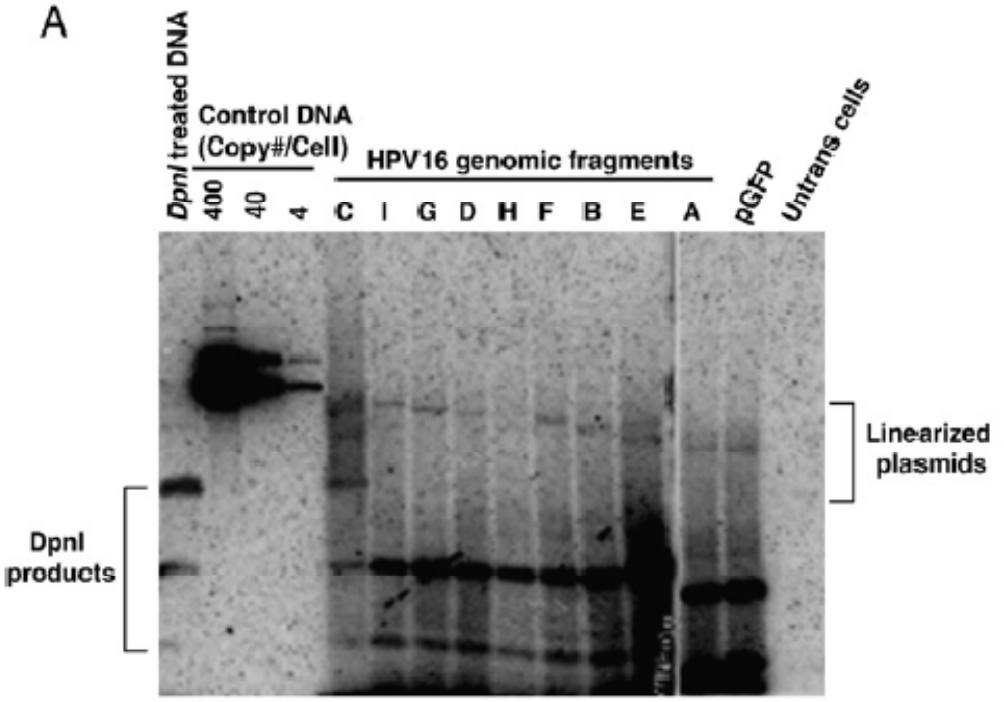

A
1
1000
2000
3000
4000
5000
6000
7000
7904
E6
E7
E1
E2
E4
E5
L2
L1
LCR
A
B
C
D
E
F
G
H
I
I
B
hARS
HPV16
subgenomic
fragments
EGFP
pGAB1
Neo^R
C
HPV16
subgenomic
fragments
EGFP
pGFP
Neo^R

Figure3. 4 Identification of regions in HPV16 that is able to substitute for replication element. (A) Short-term replication of HPV16 library constructs in pGFP vector. Hirt-extracted DNA at 4 days posttransfection was analyzed by Southern blot after overnight digestion with DpnI and XhoI. (B) The loss rates of each plasmid were determined as the relative loss rate per cell generation. The loss rates of each plasmid were compared to that of the most stable construct.


A
DpnI treated DNA
Control DNA
(Copy#/Cell)
400
40
4
HPV16 genomic fragments
C
I
G
D
H
F
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A
pGFP
Untrans cells
Linearized
plasmids
DpnI
products

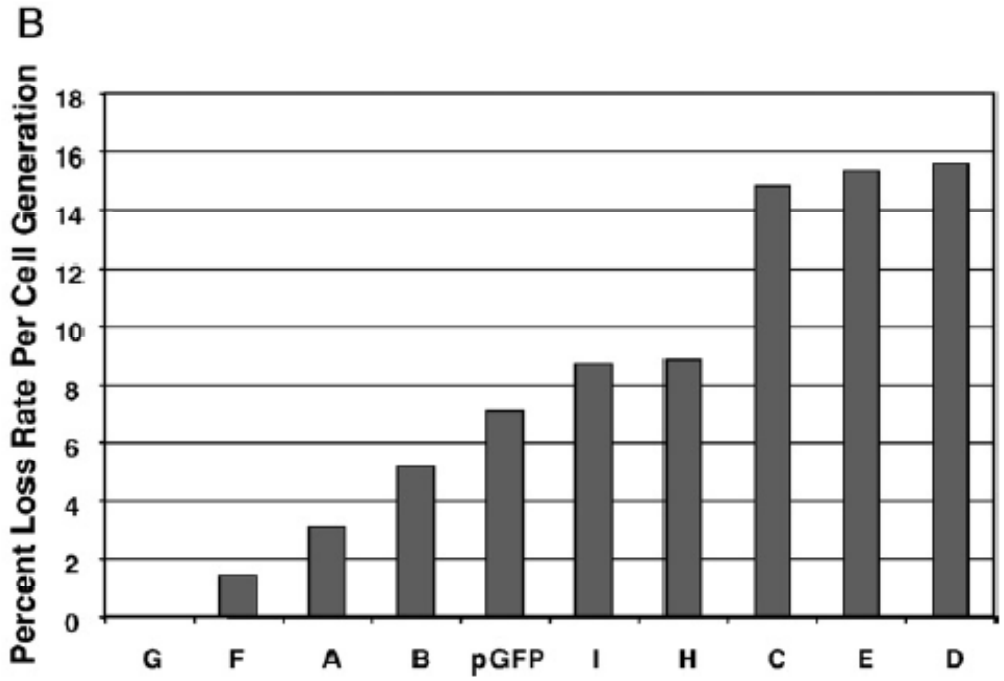
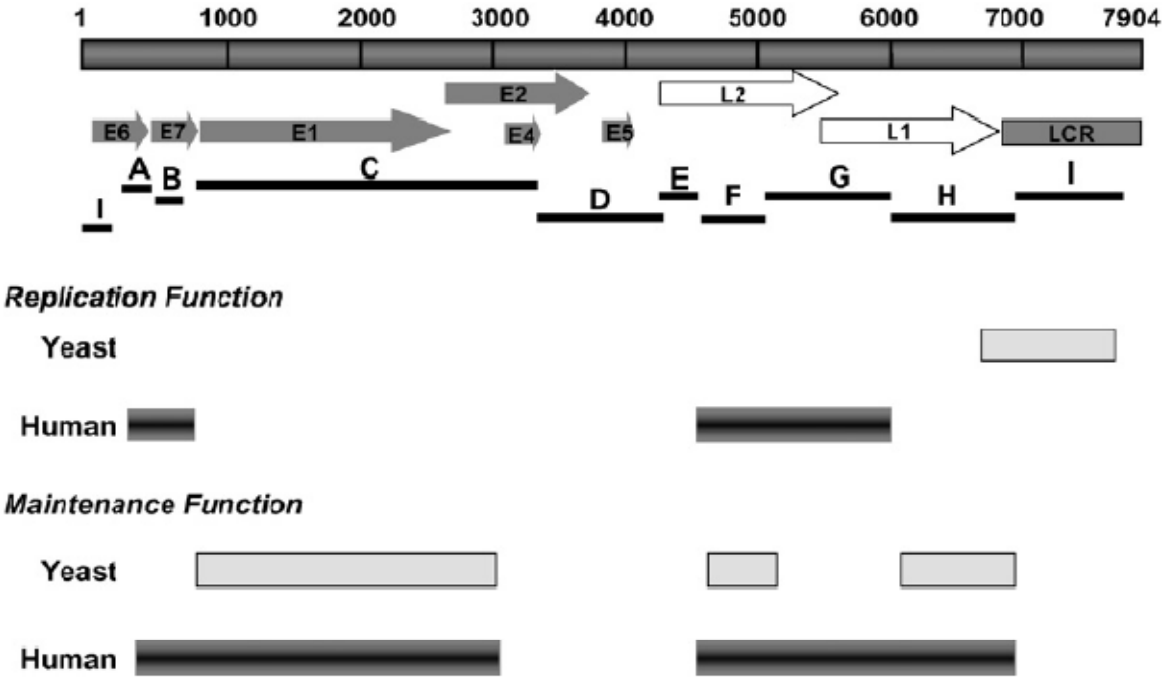

B
Percent Loss Rate Per Cell Generation
0
2
4
6
8
10
12
14
16
18
G
F
A
B
pGFP
I
H
C
E
D

Figure 3.5 Comparison of replication and maintenance elements mapped in yeast and human cells. The replication and maintenance elements mapped in yeast are shown as light gray bars. The dark beveled bars show significant replication and maintenance functions mapped in human cells in this study.


1
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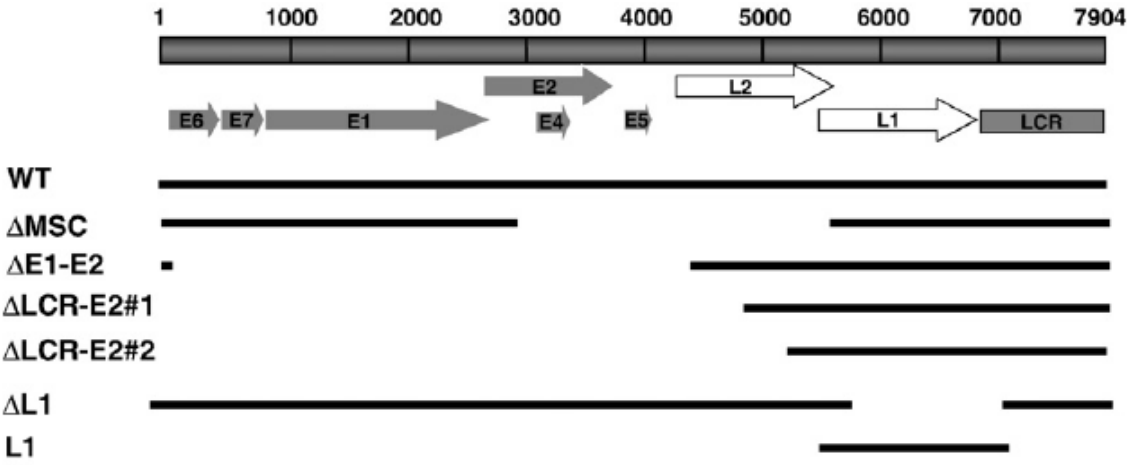
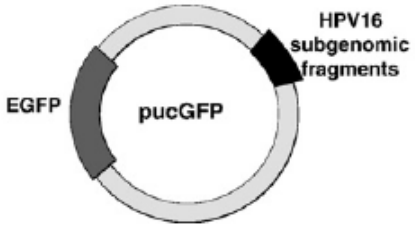
7000
7904
E2
L2
E6
E7
E1
E4
E5
L1
LCR
A
B
C
I
D
E
F
G
H
I
Replication Function
Yeast
Human
Maintenance Function
Yeast
Human

Figure 3.6 Map of the pUCGFP plasmid. pUCGFP was created from the plasmid pUC19. An expression cassette containing the EGFP gene, driven by a CMV promoter, was cloned into the multiple cloning site (MCS). The wild type HPV16 and various mutants, with respective deletions, shown in diagram, were inserted into pUCGFP at BamHI site.


1
1000
2000
3000
4000
5000
6000
7000
7904
E2
L2
E6
E7
E1
E4
E5
L1
LCR
WT
ΔMSC
ΔE1-E2
ΔLCR-E2#1
ΔLCR-E2#2
ΔL1
L1


HPV16
subgenomic
fragments
EGFP
pucGFP

# CHAPTER 4

## A Role for Telomere-related Factors in HPV16 DNA Maintenance

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**ABSTRACT**

Human papillomavirus (HPV) genomes can persist as episomes in infected individuals for years, contributing to development of cancers. Several studies have shown that bovine papillomavirus (BPV) E2 tethers newly synthesized viral genomes to mitotic chromosomes to ensure faithfully partitioning of genomes to daughter cells. However, HPV E2 proteins associate with mitotic chromosomes to a varied extent, suggesting alternate mechanisms of HPV maintenance. Our recent studies have shown that an E2-independent *cis*-acting maintenance function mapped to the L2 and L1 ORFs (nt 4538-7013). Interestingly, within the late region L2, L1, and the LCR of HPV16, we found four telomere-related factor (TRF) binding sites. Further analyses of several HPV genomes revealed that TRF binding sites are relatively common in HPV genomes and are usually found in the late region. Evidence in Epstein-Barr virus (EBV) demonstrated that TRFs and its associated proteins contribute to the viral persistence. Together with the observation that expression levels of these proteins are altered in HPV16 harboring cells, we hypothesized that these proteins have a similar influence on HPV DNA maintenance. Here we show that telomere binding proteins telomeric repeat binding factors 1 (TRF1), TRF2, protection of telomere 1 (POT1) and a RecQ DNA helicase WRN bound to the predicted TRF binding sites within the *cis*-maintenance element of the HPV16 late region. Constructs containing deletion mutants but not point mutation mutants showed effect in a long-term assay suggesting that the TRF binding sites as well as neighboring sequences may play a role on DNA maintenance in HPV16. These results imply that the telomere binding factors are novel cellular factors for HPV16 DNA maintenance.

**INTRODUCTION**

Human papillomaviruses comprise a group of small DNA viruses that infect and replicate in the stratified layers of mucosal or cutaneous epithelial cells. HPV infections are often long-lived and arguably capable of a form of latency. The viral genomes are stably maintained as low copy number nuclear plasmids in the nuclei of the proliferating cells for decades. Mechanisms ensuring the high-fidelity replication and efficient segregation of the newly synthesized viral DNA to the dividing cells are key features of successful persistence of an HPV infection. An analogy for the possible strategy of stable HPV DNA maintenance has been demonstrated for several DNA tumor viruses, such as Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), which also have similar latency stages in their life cycles (Ballestas and Kaye, 2001, Barbera *et al.*, 2006, Harrison et al., 1994, Lupton and Levine, 1985, Skalsky et al., 2007, Yates et al., 1984). Hitchhiking of viral DNA along with mitotic chromosome, by use of a virally encoded tethering protein to allow partitioning and nuclear retention of the viral genomes appears to be a favored mechanism among persistent viruses (Botchan, 2004, Feeney and Parish, 2009). In all examples, a single viral protein acts as a molecular linker to host mitotic chromosomes. The viral transactivator EBNA1 and transcriptional repressor LANA1 are the linkers used to serve this hitchhiking strategy in EBV (Lindner and Sugden, 2007)and KSHV (Ballestas et al., 1999, Ballestas and Kaye, 2001), respectively.

In the case of papillomaviruses, a similar mode of viral DNA maintenance has been characterized, mainly based on studies related to chromosomal segregation in bovine papillomavirus type 1 (BPV1). The genome tethering function is mediated by the multifunctional viral E2 protein, which is composed of an N-terminal transactivation domain and a C-terminal DNA binding domain, linked by a nonconserved flexible hinge. E2 protein mediates

the genome maintenance by interaction of its transactivation domain with mitotic chromosome associated protein, while the DNA binding domain tethers genomes through an association with multiple E2 binding sites. Further studies have identified that Brd4, a cellular bromodomain protein, is a major component of the tethering complex that attach the viral genomes to the host mitotic chromosomes (Baxter et al., 2005, McBride et al., 2004, McPhillips et al., 2005, You et al., 2004). Both the BPV1 E2 protein and viral genomes are colocalized with Brd4 on mitotic chromosomes in punctate spots with no specific attachment sites apparent. Disruption of the E2-Brd4 interaction dissociates E2 from chromosomes and abolishes the viral genome maintenance (McBride et al., 2004). Although this interaction has been shown to be the mechanism of DNA maintenance for BPV1, it appears that in other PV types that the interaction between E2 and Brd4 is required for E2-mediated transcriptional regulation, rather than necessarily for genome tethering (McPhillips et al., 2006, Schweiger et al., 2006, Wu and Chiang, 2007). Further analysis has demonstrated that HPV8 or HPV18 E2 mutants lacking Brd4 binding remained attach with mitotic chromosome (McPhillips et al., 2006). Therefore, different PVs might utilize different cellular proteins or alternate strategies to maintain the viral genome in the host cells.

Because the viral genome tethering function of the E2 protein has been well established in BPV1, the attachment of PV E2 proteins to mitotic chromosome was also analyzed and found to be conserved in several cases, such as HPV1a and other animal PVs. However, the HPV8 E2 was found to interact with the pericentromeric region that contains β-satellite DNA and the ribosomal DNA (rDNA). The RNA polymerase I transcription factor UBF1 was recently identified as a potential cellular partner for HPV8 E2 (Poddar et al., 2009). In further studies, the E2 proteins from human α papillomaviruses HPV11, HPV16 and HPV31 were found not to stably

associated with chromosomes throughout mitosis process (Oliveira et al., 2006). They showed localization with the mitotic chromosome only under certain prefixation treatments, indicating a weak affinity of these $\alpha$ papillomaviruses E2 for the chromosomes (McBride et al., 2004). In separate studies, the $\alpha$ papillomaviruses HPV11, HPV16, and HPV18 were found associated with mitotic spindles instead of the chromosomes (Van Tine et al., 2004). The observation that only 37% similarity of E2 shared among papillomaviruses and the variation in the association of different PV E2 proteins with mitotic chromosomes raised the intriguing possibility E2 protein might mediate partitioning of the human papillomavirus DNA. The mechanism for genome maintenance in HPVs remains elusive, and may require multiple strategies, thus require investigation.

The role of *cis*-elements in genome partitioning has been investigated in BPV1. The minimal requirement for BPV1 genome maintenance has been mapped to the LCR containing 11 E2 binding sites. At least ten of these binding sites, referred to as minichromosome maintenance element (MME), are required in this process. Most $\alpha$ papillomaviruses contain only four consensus E2 binding sites and it not yet clear whether these would be sufficient for HPVs DNA maintenance using a similar mechanism to BPV1. To ensure the DNA partitioning in dividing cell during mitosis, it is likely that these viruses use different chromosomal targets and approaches to stabilize their genomes. One potential possibility is an alternate *cis*-element that binds distinct cellular or viral proteins and attaches the genomes to cellular mitotic chromosomes.

In previous studies, we have mapped maintenance elements that provide plasmid stability to two distinct regions in HPV16 genome. These HPV16 subgenomic fragments are located outside of the LCR that contains multiple E2BS and they function as maintenance

elements in the absence of any viral protein. This finding is in concordance with our recent studies using yeast, in which subgenomic fragments of HPV DNA can autonomously replicate and persist independently of E1 and E2 proteins (Piirsoo et al., 1996). In other experiments, we demonstrated that HPV16 genome mutants containing a disrupted E2 gene, are still stably maintained as episomes in human cells. Together, these studies led us to speculate that the interaction of viral cis-elements with cellular factors may influence HPV DNA maintenance.

Our sequences analysis identified several possible cellular candidates that potentially could bind to the viral genome and contribute to HPV DNA maintenance. Those cellular proteins are high mobility group (HMG), topoisomeraseII (TopoII), centromere binding protein (CENP-B), nuclear matrix attachment region (MAR/SAR) proteins, and telomere related factors (TRFs). Of those potential candidates, four TRF binding sites are found within the viral genome and three of which are located within the previously mapped *cis*-acting maintenance element (Fig.4.1A). Further, TRF binding sites are prevalent among many HPV genomes whereas there are none in BPV1. For EBV, three nonamer sites (TTAGGGTTA), responsible for TRF cognate sequence, contribute to plasmid maintenance and DNA replication function (Atanasiu et al., 2006, Deng et al., 2002). The regulation of DNA maintenance was mediated by an interaction of TRF2 proteins with these binding sites in OriP containing plasmids. Motivated by these observations, we hypothesize that the TRF binding sites, and TRF proteins contribute to episomal maintenance of HPV genome.

Telomere-related factors, TRF1 and TRF2, are regulators of telomere length and required to maintain cell viability (Gilson and Geli, 2007, Smogorzewska and de Lange, 2004). The ratio of TRFs and related proteins such as POT1 and TRF2 interacting protein RAP1 as well as the interplay between these factors may determine the growth status of the cells. Alteration of

these proteins has implication on cancer progression and cell regulation (Cookson and Laughton, 2009). TRFs may also be considered as segregation factors since they are able to associate with nuclear matrix and function in separating telomeres during mitosis (Ermler *et al.*, 2004, Luderus *et al.*, 1996).

In these studies, we employed supershift assays and chromatin Immunoprecipitation assays (ChIP) to examine the interaction between telomere-related proteins and suspected TRF binding sites located in the HPV16 genome. We found that TRF2 could bind to the predicted TRF binding sites in the viral genome. TRF2 was originally characterized as a telomere repeat binding that promotes T-loop formation in vitro to protect chromosomal ends from end-to-end fusions and from nuclease degradation (Stansel et al., 2001, Yang et al., 2005). TRF2 is a member of myb family of DNA binding domains and it binds to telomeric DNA by means of the Myb-like motif. TRF2 was also interact with a single-stranded telomeric DNA binding protein POT1 to maintain telomere intregity (Yang et al., 2005). Interestingly, TRF2 and POT1 proteins physically bind to RecQ helicase, WRN and thereby stimulate helicase activity to unwind duplex telomeric substrate (Gilson and Geli, 2007). The precise role of RecQ helicases in telomere maintenance is still unclear, however, they likely function in recombination and replication of telomeric ends.

Since TRF2 has several binding partners, and the expression level of these proteins is altered in HPV16 harboring cells, we suspected that this alteration may correlate with differences in episomal maintenance of the HPV genome. In this work, we have used ChIP assay examine the interaction of TRF2 and its related proteins with the predicted TRF binding site in HPV16 DNA. We also investigated the role of the suspected TRF binding sites and the associated proteins in the viral DNA maintenance. It appeared that telomere related proteins such as TRF2,

POT1, and WRN can bind to the HPV16 genome at the TRF binding site and such interaction may contribute to the regulation of the viral genome stability. However, further investigation requires to be done in order to reveal precise role of these cellular proteins on the viral life cycle.

## MATERIALS AND METHODS

### *Cell culture and transfection method*

Human embryonic kidney 293 cells and two genetic variants stably expressing Epstein Barr nuclear antigen 1, EBNA1, (293E) or the large-T antigen (293T), the cervical cancer cell line (HeLa) and spontaneously transformed human keratinocytes (HaCat) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlas, USA) and 1 mM sodium pyruvate. Osteoblast cell line that stably expresses HPV16 E2, U2OS16E2, and its parental cell line, U2OS, were cultivated in DMEM containing 10% FBS. Primary cells, neonatal human foreskin keratinocyte (NHFK), human foreskin keratinocyte (HFK) and their derivative cells, HPV16 transformed NHFK (HPV16NHFK) and hTERT-immortalized HFK (hTERT-HFK) were maintained in Keratinocyte Growth Medium-2, KMG-2, supplemented with KGM-2 Bullet Kit (Lonza, USA) and 0.075 mM calcium chloride. All cells are cultured at 37°C in a fully humidified atmosphere of 5%$CO_2$.

Primary keratinocytes were transfected using Primefect (Lonza, USA). Other cell lines were transfected with Dreamfect according to the manufacturer's protocol. Briefly, cells were plated the day before transfecting at density of 4 x $10^5$ per 60 mm dish or 2 x $10^6$ per 100 mm dish. Cells were transfected with 2 μg (60 mm dish) or 5 μg (100 mm dish) of DNA in DMEM containing a ratio of 4:1 Dreamfect (μl):DNA (μg) or 10:1 Primefect (μl):DNA (μg).

***Plasmids***

Plasmid pEF399 contains an entire HPV16 genome in pUC19 backbone and was described previously by Kitai K et al (Kim et al., 2005). Plasmid 2380 which contains wild-type EBV OriP cloned in pPUR was a gift from Dr. Paul Lambert (University of Wisconsin-Madison). Plasmid 2380.1 was constructed as a derivative of 2380, by inserting the AflIII fragment from pEGFP C1, which contains an expression cassette of enhanced green fluorescent protein (EGFP) gene, into the EcoRI site of 2380. The EcoR I treated 2380 DNA ends were blunt-ended by use of DNA polymerase I (klenow) prior to ligation. Expression of EGFP is driven under the control of the cytomegalovirus (CMV) promoter. Plasmid 2380.2 has a 914 bp deletion between Mlu I and Xho I from the plasmid 2380.1, resulting in the removal of the family of repeat (FR). Subgenomic fragments of the HPV16 late region containing 0, 1, 2, and 3 predicted TRF binding sites were then inserted in place of the FR to generate derivative constructs 2380.3-2380.9 (Fig 4.4A). Those individual fragments were cloned into 2380.2 using multiple linkers shown as follows; linker PEDB Top, 5'-GTGCTAATAGAATCCTTACCGAGGCACTTCGTGG-3'; PEDB bottom, 5'-GATCCCACGAAGTGCCTCGGTAAGGATTCTATTAGCAC-3'; linker SM top, 5'-CGTTCTTGCTCTCGTTACACA-3'; linker SM bottom, 5'-CGCGTGTGTAACGAGAGCAAGAACGCATG-3'; linker PK top, 5'-GTGGTTCTTGCTATCGGGCATGGTAC-3', linker PK bottom, 5'-CATGCCCGATAGCAAGAACCAC-3'; linker PXPM top, 5'-CTGAAGTCGTTCCACGTGGAACTAATATA-3'; linker PXPM bottom, 5'-CGCGTATATTAGTTCCACGTGGAACGACTTCAG-3'.

The 2380.3 contains nucleotides 4140-7464 of HPV16 inserted in between PmlI and MluI sites of 2380.2 with the linker PXPM. The 2380.4 construct was made by ligation of HPV16 nucleotides 4906-7464 into the PmlI site of 2380.2. The 2380.5 was a result from insertion of nucleotides 4466-7464 of HPV16 genome along with the linker SM into 2380.2 at Pml I-Mlu I

sites. The 2380.6 contains HPV16 nucleotides 5377-7464 cloned into Pml I-Mlu I sites of 2380.2 with the linkers PK and SM. The 2380.7 has HPV16 nucleotides 6150-7464 as well as the linkers PEDB and SM placed in between PmlI and Mlu I sites of 2380.2. The 2380.8 was generated by deleting the Dra III fragment of 2380.7 and thus left HPV16 nucleotides 6517-7464 in it. The last constructs, 2380.9, contains nucleotides 6517-7464 of HPV16 that was made by eliminating the EcoNI fragment of 2380.7.

To generate TRF mutants, the construct 2380.5 containing the HPV16 late region (nucleotides 4466-7464) that harbors three predicted TRF binding sites was modified by site-directed mutagenesis (Stratagene Quickchange Kit, Stratagene) with 6 bp substitutions converting the site(s) to MluI (Fig .4.5A). The predicted TRF binding sites were designated A, B, and C by the order of their distributions in HPV16 genome (Fig. 4.1). Initially, sites A, B, and C in plasmid 2380.5 were individually altered to make single TRF binding site mutation, referred to as 2380.5ΔA, 2380.5ΔB and 2380.5ΔC. These single-site mutants were then further mutated to obtain double- and triple-site mutations. Constructs with two TRF binding sites changed to Mlu I were named as 2380.5ΔAB, 2380.5ΔAC, and 2380.5ΔBC, according to the sites that were modified. The triple-site mutation mutant that harbors all three TRF binding sites converted to Mlu I recognition sequences was denoted as 2380.5ΔABC.

***Nuclear extracts***

Nuclear extracts were prepared at 4°C by extraction of nuclei with high salt buffer by the following method. Briefly, cells were lysed with NP40 lysis buffer (50 mM NaCl, 10 mM HEPES pH 8.0, 500 mM Sucrose, 1 mM EDTA and 0.5% NP40). Cell nuclei were washed afterward with low salt buffer containing 50 mM NaCl and then extracted with high salt buffer (350 mM NaCl, 10 mM HEPES pH 8.0, 25% glycerol, 0.1 mM EDTA). All buffers were freshly

added with 7 mM mercaptoethanol and 1X protease inhibitor cocktail III (Calbiochem) before used.

***Electrophoretic mobility shift assay (EMSA)***

Five pmoles of double-stranded DNA probe were end-labeled with $^{32}P$ by T4 polynucleotide kinase. The labeled probe was incubated with crude nuclear extract (typically 1 μl with ~ 7 μg of protein) in a volume of 20 μl of EMSA buffer (20 mM HEPES-KOH pH 8.0, 150 mM KCl, 1 mM $MgCL_2$, 0.1 mM EDTA, 0.5 mM DTT, 5% glycerol, 4% Ficoll [molecular weight 400.000]) with 0.5 to 1 μg of sonicated E. coli chromosomal DNA. The mixture was carried out at room temperature for 30 min. For supershift assays, 2 μg of antibody were added to the reaction for another 20 min. The DNA-protein complexes were then fractionated on a 5% polyacrylamide gel in 1X TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) at 50-60 mA for 2.5 h at 4°C. Gels were dried under vacuum at 70-80°C. The dried gels were exposed to a phosphoimager screen overnight and analysis was performed using Bio-Rad Molecular Imager FX. The probes in EMSA and supershift experiments are described as follows. TRF binding site A top, 5'-TGTTAGCACAAACCCTAACA-3'; TRF binding site A bottom, 5'-TGTTAGGGTTTGTGCTAACA-3'; TRF binding site B top, 5'-GTCCACCATTAGAGTTAATA-3'; TRF binding site B bottom, 5'-TATTAACTCTAATGGTGAC-3'; TRF binding site C top, 5'-TGTGCAAAATAACCTTAACTGC-3'; TRF binding site C bottom, 5'-GCAGTTAAGGTTATTTTGCACA-3'; TRF binding site D top, 5'-CCGTTTTGGGTTACACATTT-3'; TRF binding site D bottom, 5'-AAATGTGTAACCCAAAACGG-3'. The predicted TRF2 binding sites A, B, C, and D are underlined.

***Chromatin immunoprecipitation assay (ChIP)***

Human embryonic kidney cells, 293 were transfected with the construct, pEF399, which contains an entire HPV16 genome, using Dreamfect (Boca Scientific). At 4 days after the transfection, approximately 2 x $10^7$ cells were harvested and fixed with 1% formaldehyde at room temperature for 10 min with gently shaking. The fixation was subsequently quenched by addition of glycine to a final concentration of 0.125 M. After 10 min at room temperature, the cells were pelleted, washed twice with ice-cold PBS and lysed on ice in cell lysis buffer containing 5mM PIPES (pH 8.0), 85 mM KCl, and 0.5% NP40 for 10 min. After cell lysis, nuclei were pelleted and partially lysed by quick freezing in liquid nitrogen and thawing on ice. Micrococcal nuclease (MNase, USB) digestion was performed for 10 min at 37°C in a reaction containing MNase buffer (10 mM Tris [pH 7.5], 1 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM NaCl, 4% NP40, 1 mM PMSF, and protease inhibitor cocktail III [Calbiochem]) and 50 U. The MNase reaction was terminated by sequential addition of 3 mM EGTA and 1%SDS. After incubation on ice for 10 min, the solution was sonicated 30 seconds 3 times with 1-min pause interval. The sonicated sample was centrifuged at maximum speed at 4°C for 30 min. An aliquot (~1/10 of supernatant) was taken as control to check quality of DNA. Five hundred µl of the extract was diluted 1:10 with ChIP dilution buffer (16.7 mM Tris-HCl, pH8.0, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS). The diluted solution was precleared with Protein G Dynabeads (Invitrogen) which were preblocked with 0.5 mg/ml sonicated bacterial DNA and 1 mg/ml BSA. After 1 h incubation at 4°C, approximately, 1/10 of precleared supernatant was taken as an input DNA control and the remaining solution was then equally divided and incubated separately with a specific antibody, mouse IgG (as control antibody) or no antibody (as mock Immunoprecipitation) at 4°C overnight. Antibody used were anti-TRF1 (Santa Cruz), anti TRF2

(Santa Cruz), anti-POT1 (Abcam), Anti-WRN (Novus Biologicals), anti-BLM (Santa Cruz), anti-TIN2 (Santa Cruz), or control mouse IgG (Invitrogen). Preblocked protein G Dynabeads were then added, and the mixture was incubated at 4°C for 2 h to pull down immunoprecipitate complexes. The beads were washed withff; low salt immune complex wash buffer (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS); high salt immune complex wash buffer (20 mM Tris-HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS); LiCl immune complex wash buffer (10 mM Tris-HCl, pH 8.1, 0.25 mM LiCl, 1 mM EDTA, 1% deoxycholic acid, 1% IGEPAL-CA630) and TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The DNA-protein complexes were then eluted twice with 250 μl of elution buffer (0.1 M $NaHCO_3$, 1% SDS). Twenty μl of 5 M NaCl were added to input control and the eluted supernatant containing immunoprecipitate complexes and incubated at 65°C overnight to reverse cross-linking. Immunoprecipitated DNA was then purified with phenol:chloroform:isoamyl alcohol (25:24:1), chloroform:isoamyl alcohol (24:1) and precipitated with 40 μg/ml glycogen and isopropanol at room temperature for 20 min. After precipitation, DNA was washed once with 70% ethanol, vacuum dried and resuspended in 30 μl of water. The purified DNA was subjected to PCR for TRF binding sites (A, B, C, and D) or HPV16 early region (as negative control). PCR primers were as follows: TRF binding site A forward, 5'-ACCCATCTGTATTGCAGCCTC-3'; TRF binding site A reverse, 5'-AACTTGTTGTGTTGTGCGAC-3'; TRF binding site B forward, 5'-GTCGGTACCCCATGTACCAATGTTGCAG-3'; TRF binding site B reverse, 5'-CACCTGGATTTACTGCAACATTGG-3'; TRF binding site C forward, 5'-AGCACAGGGCCACAATAATG-3'; TRF binding site C reverse, 5'-TCTTCTAGTGTGCCTCCTGG-3'; TRF binding site D forward, 5'-CTAAGGCCAACTAAATGTCACC-3'; TRF binding site D reverse, 5'-CGATTTCGGTTACGCCCTTAG-3'; HPV16 early region forward,5'-TACGACGCGTGATCAGAGCCAGACAC-3'; HPV16 early region reverse, 5'-CGATGGATCCCATCACCTTTTAAATGTACT-3'. The PCR products were resolved on a

1.5% agarose gel and visualized after ethidium bromide staining. Gel images were digitally captured by use of a Fluor-S-Multiimager (Bio-Rad), and the band intensities were quantified by using Quantity one software (Bio-Rad). The ChIP assay was performed in HPV16 immortalized NHFK without plasmid transfection.

***Plasmid Stability Assay***

Approximately 4 x $10^5$ 293E cells were transfected with 2 μg of plasmid DNA using the dreamfect reagent. The media was removed at 1 day posttranfection. On the next day, the transfected cells were cultured in the presence of 1 μg/ml of puromycin for 4 days to favor the growth of cells containing the plasmid,and select out untransfected cells. After selection, cells were grown for another 45 days. During the experimental period, cells were maintained and passaged every 4-5 days. Low molecular weight DNA was isolated by Hirt extraction from equal numbers of cells. Dpn I digestion was performed overnight at 37°C along with the single-cutting enzyme, XcmI to generate linear DNAs and eliminate bacterially methylated input DNA. The digested samples were subjected to Southern analysis using ampicilin-specific probe that was labeled with [$^{32}$P]dCTP by random prime labeling system (GE Healthcare). Radioactive signals on the blots were visualized by autoradiography and were quantified on PhosphorImager using Quantity one software (Bio-Rad).

***Western blotting***

Primary antibodies to TRF1 (GeneTex), TRF2 (GeneTex), POT1 (Abcam), hRap1 (Lifespan Bioscience), hTERT (Santa Cruz), TIN2 (Santa Cruz), TPP1 (R & D Systems), WRN (Novus Biologicals), BLM (Santa Cruz), and histone H3 (Cell Signaling Technology) were purchased and used according to manufacturer's protocols. Basically, 30 μg of nuclear or whole cell extract

were separated through 10% SDS-PAGE gels. Proteins were transferred to Immobilon-P membrane (Millipore). The membrane was blocked for 1 h with Tris-buffered saline/Tween (0.02 M Tris-HCl, pH 7.6, 0.136 M NaCl, 0.1% Tween 20) (TBS-T) containing 5% nonfat dry milk and incubated with primary antibody at 4°C overnight. Primary antibodies were diluted 1:1,000 or 1:2,000 in the blocking solution (TBS-T plus 5% dry milk). Blots were washed three times for 10 min each with TBS-T, incubated with appropriate secondary antibody conjugated with horseradish peroxidase, developed with Amersham ECL reagents and then exposed to X-ray films.

***Short-term DNA replication assay***

The DpnI-based transient replication assay was performed as previously described with some modifications (Wang *et al.*, 2006) to measure the plasmid replication efficiency in short term. Briefly, cells were transfected with plasmid DNA using Dreamfect following the manufacturer's instructions. At 4 days after transfection, equal numbers of cells ($1 \times 10^7$ cells) were harvested and extracted for plasmid DNA by the Hirt method (Hirt, 1967). Prior to the isolation of Hirt DNA, 10 ng of pET21a (+) was spiked into each sample to monitor DNA recovery and completeness of DpnI digestion. Approximately, 1/10 of the Hirt DNA samples were digested with a single-cutting enzyme, Xcm I, at 37°C for 4 h. The remaining sample was sequentially digested overnight with DpnI after 4 h digestion with XcmI. The digested DNA samples were then separated on a 0.8% agarose gel and subjected to Southern analysis using ampicillin-specific probe. The probe was labeled with [$^{32}$P]-$\alpha$dCTP by use of a random primer labeling kit (GE Healthcare). Autoradiographs were captured by use of a PhosphoImager and Quantity one software (Bio-Rad).

***Flow cytometry analysis***

EGFP expression was analyzed by Flow cytometry to measure the loss of plasmid DNA in long- term culture. EGFP expression data were collected on days 0 and 12 after selection was removed. The rate of plasmid loss was calculated by use of following formula: rate of loss per cell generation = $[1- (F/I)^{1/N}]$ [100], where I is the initial percent EGFP positive cells under nonselective conditions and F is the percent EGFP positive cells after n generations. N is the number of cell generations in the absence of selection. The rates of loss per cell generation were calculated from duplicate data sets.

**RESULTS**

***Expression of telomeric DNA binding proteins and related proteins in various cells***

Many studies have shown that hTERT is up-regulated in high risk HPV infected cells by E6 and E6AP mediated process. Subsequent elevated expression of hTERT in these cells imparts a tumorigenic phenotype, presumably through the maintenance of telomere length. The regulation of telomere integrity involves not only the hTRET but alos several proteins associated with telomeric repeat unit (TTAGGGTTA) at the chromosome ends. Therefore, induction of hTERT by E6 protein might disturb expression of the protein associated with telomeric DNA. However, expression levels of telomere related proteins have not been considered in the context of tumorigenesis. Therefore, we examined the expression profiles of telomere related proteins in various transformed cells, included HPV-harboring cells, compared to normal cells. Cells used in this study included virally or spontaneously transformed cells derived from different cell types, as well as normal primary keratinocyte cells. Those transformed cells are 293 cells; human embryonic kidney cells transformed by sheared adenovirus 5 DnA and its variant 293T which express SV40 T antigen, HeLa; cervical cells containing integrated HPV18

genome, HaCat; spontaneously transformed human keratinocyte cells, U2OS; hTERT defective cells derived from osteoblastoma and its derivative U2OS 16E2 which expresses E2 protein from HPV16, hTERT-HFK; hTERT-immortalized human foreskin keratinocyte, and HPV16-NHKF; HPV16 immortalized neonatal human foreskin keratinocyte. Primary normal cells isolated from foreskin keratinocytes, NHFK and HFK, were used as controls.

To assess expression of hTERT and other associated proteins, equal amount of whole cell extracts (30 μg) from transformed cells and normal cells were subjected to SDS-PAGE followed by Western blot analysis with specific antibodies. Coomassie gel staining in the left bottom panel of Fig. 4.2B demonstrated approximately the same amount of total protein loadings. The results showed that hTERT, TRF1, and TRF2 are expressed at higher levels in transformed cells 293, 293T, HeLa, and HaCat compared to normal keratinocyte cells. In contrast to hTERT and TRF proteins, other related factors such as POT1, TIN2, RAP1 and WRN stayed at the same levels as those in normal cells (Fig 4.2B).

We also investigate expression levels of telomere binding proteins and associated partners in keratinocytes that are immortalized by HPV16 genome or hTERT gene compared to their parental normal counterparts. The HPV16 transformed keratinocytes was established by transfection of primary foreskin keratinocytes, NHFK with the HPV16 genome containing plasmid. The transfected cells that went through senescence crisis (more than 30 passages) and became immortal were selected and referred as to HPV16 immortalized cells (HPV16-NHFK). The presence of HPV16 genome in HPV16-NHFK cells was detected by Southern analysis using radiolabeled HPV16 DNA as a probe (Fig.4.2A). The hTERT-immortalized keratinocytes were a gift from Dr.Al Klingelhutz. In the Western blot analyses, we found that TRF2 was up-regulated in HPV16- and hTERT- immortalized cells, compared to their normal parental cells. No notable

differences in TIN2 and POT1 expression levels in transformed cells was observed, whereas RAP1 was clearly down-regulated in both immortalized cells. We also observed that WRN helicase levels were elevated in hTERT-immortalized cells, to a modest degree.

***In vitro binding of telomere associated proteins to putative TRF binding sites on HPV16 genome***

Based on TRF consensus binding sequences (TTAGGGTTT), we found that HPV16 DNA contains four suspected TRF binding sites were mostly located within the late region of HPV genomes. Three of such TRF binding sites (A, B, and C) lie within the L1 and L2 ORFs, which was defined as for having maintenance function, while the TRF binding site D at the LCR region is overlapping with one of the E2 responsive binding site (Fig 4.1). We recognized by analogy that the spacing of these TRF binding sites within HPV16 resemble the dyad symmetry (DS) nonamers in EBV that were found to associate with TRF2 and other TRF-related proteins. However, each predicted TRF binding site harbors one mismatch sequence as shown in Fig 4.1 and no evidence, until our work, demonstrated that these sites interact with telomeric repeat binding factors. To determine whether telomere-binding factors associate with the HPV16 genome, we first performed gelshift analysis using 293 nuclear extract. We further identified the proteins that formed a complex with the TRF binding sequences by a supershift assay using antibodies specific to six proteins (TRF1, TRF2, POT1, RAP1, TIN2, and TPP1) that formed a core complex with TRFs at telomeric DNA sequences (Songyang and Liu, 2006). The results showed that TRF2 was bound to the TRF binding sites A, B, and D in HPV DNA but not to a great extent to binding site C (Fig 4.3). Although our supershift data failed to show any significant binding of other telomere-related proteins, we suspect that these proteins may participate in this complex, but their

interactions may be dynamic, and thus difficult to detect by gelshift assay. It is also possible that other viral proteins could facilitate the interaction, for example E2.

***Binding of TRFs and associated proteins to HPV16 in cells***

Chromatin Immunoprecipitation (ChIP) assay was performed as an alternative approach to demonstrate the interaction of telomeric DNA binding proteins and predicted TRF binding sites on HPV16 genome in cells. Human embryonic kidney cells, 293 were transfected with the plasmid, pEF399 which contains an entire HPV16 genome. At 4 days posttransfection, the transfected cells -were lysed and crosslinked with formaldehyde. Protein-DNA complexes were sheared into small fragments using MNase digestion followed by sonication prior to be immunoprecipitated with specific antibodies. The sheared DNA showed optimal size range for immunoprecipitation, approximately 100-1,000 bp with the majority of DNA fragments between 100-300 bp (Fig.4.4B). Multiple sets of primers employed in PCR reaction were shown in Fig.4.4A. As a positive control, input DNA was used to amplify TRF binding sites A, B, C, and D or the E2 sequence in the early region of HPV16 located over 2 kb away and unlikely to bind to telomeric factors (Fig.4.4B, lanes 1 and 2). The amplification of TRF binding site and E2 sequences from input DNA yielded the same amount of PCR products whereas these HPV16 sequences were not amplified from mock-tranfected cells (Fig.4.4B, lane 19).

As shown in Fig.4.4B, antibodies specific for POT1 and WRN efficiently precipitated theTRF binding sites A, B, and D (Fig.4.4C lanes 8, and 14) which were undetectable in the immunoprecipitant with no antibody or control mouse IgG (Fig.4.4C, lanes 15-18). These three binding sites were also precipitated by anti-TRF2 antibody with less efficiency. The E2 sequences in HPV16 early region was not precipitated with any antibodies specific for telomere

repeat proteins (Fig.4.4C, bottom panel), demonstrating that these telomere proteins, TRF2, POT1, and WRN specifically bind to the putative binding sites on HPV16 in vivo.

We also further extended the ChIP assays to another human cell line, HPV16-NHFK, the HPV16 immortalized human foreskin keratinocytes that was shown to harbor episomal HPV16 genome (Fig.4.2A). Input DNA taken from HPV16-NHFK showed a signal of HPV16 DNA inside the cells (Fig.4.4D, lanes 3 and 4) whereas it was not detectable in NHFK (Fig.4.4D, lanes 1 and 2). However, the antibodies specific for telomere repeat proteins, TRF1, TRF2, POT1, and WRN failed to pull down the HPV16DNA from the crosslinked chromatin of HPV16-NHFK cells (Fig.4.4D, lanes 5-14). Since the HPV16-NHFK maintained the HPV16 genome at very low copy number (~2 copies/cell, Fig.4.2A), it is possible that the amount of episomal HPV16 DNA in the cells is below the limit of detection by ChIP analysis.

***Implication of TRF binding sites in HPV16 DNA maintenance***

Previous studies in EBV have shown that telomeric repeat binding proteins, TRF1 and 2 bound to nanomer repeats (TTAGGGTTA) within the DS element of oriP. The mutation of nonamer sequences or the expression of dominant negative truncation mutant TRF2 (DNT-TRF2) inhibited oriP replication activity. TRF2 and other telomeric proteins may also contribute to plasmid retention during mitosis (Atanasiu et al., 2006, Deng et al., 2003, Deng et al., 2002). Interestingly, our studies also showed that TRF proteins and associated partners, POT1 and WRN can interact with the predicted TRF binding sites in the HPV16 genome and these putative binding sites are located within the HPV16 subgenomic region we had mapped for maintenance function.

To investigate the physiological role of these TRF binding sites in the HPV16 genome maintenance, we applied the well-established EBV replication and maintenance system in this study. The minimal replicator for EBV episomal DNA maintenance has been identified in OriP that contains two distinct *cis*-elements responsible for separate processes; replication and partitioning. DS confers EBNA1-dependent replication whereas FR mediates partitioning (Yates et al., 1984, Yates et al., 2000). The replication and partitioning elements were separated to construct hybrid origins which contain the DS element of EBV OriP and HPV16 subgenomic fragment with varying numbers of TRF binding sites in the background of the pPur plasmid (Fig.4.5A). The replication function of the chimeric EBV origin-based plasmids is provided by DS sequences in an EBNA-1-dependent manner while efficient partitioning would be facilitated by HPV16 sequences and telomeric repeat proteins. Similar hybrid orgin system have successfully been utilized to identify *cis*-element of other DNA tumor viruses (Silla *et al.*, 2005, Skalsky et al., 2007).

Starting with the plasmid 2380.1 containing intact wild-type EBV oirP, the FR element was replaced with either HPV16 subgenomic segment harboring three TRF binding sites or deletion mutants containing none, one or two TRF binding sites (Fig.4.5A). 293 cells stably expressing EBNA1 (293E) were transfected with each hybrid origins and either 2380.1 (intact EBV OriP) or 2380.2 (EBV OriP without FR) as controls. After transfection, cells were grown in the presence of puromycin to select for plasmid containing cells. After 4 days under a selective condition, outgrowing colonies were released from drug selection and grown in the absence of the drug for another 15 days. The plasmid retention was monitored by flow cytometry at days 0 and 15 after drug removal. The efficiency of plasmid maintenance was calculated as the rate of plasmid loss per cell generation (Fig.4.5B). As expected, the plasmid containing an intact EBV

oriP (2380.1) has two times lower loss rate than that of the one lacking FR (2380.2). Interestingly, the hybrid origin with HPV16 subgenomic fragment containing 3 binding sites, 2380.5 was lost at similar rate to the oriP plasmid 2380.1. However, we did not observe strong correlations of plasmid retention activity with the numbers of TRF binding site since high loss rate was also observed in the plasmid 2380.3 that harbors three TRF binding sites as well as in 2380.8 that contains only a single TRF binding site. One possible explanation for this variation is that sequences outside of the tRF binding site may contribute to efficiency of plasmid stability. We determined that a limitation to these experiments was that deletion of the HPV L1 sequence also removes the context in which the TRF binding sites exist. This, we believe, could interfere with our ability to determine the effect of TRF binding-sites on HPV maintenance. This phenomenon of dependence of activities of *cis*-maintenance elements on neighboring sequence context has been previously observed in EBV and KSHV (Hebner *et al.*, 2003, Skalsky et al., 2007).

To rule out the potential effects of neighboring sequence and plasmid size on DNA retention and stability, we decided to create another set of TRF mutants by either single or multiple mutating TRF binding sites in the plasmid 2380.5 that showed high ability of plasmid retention. The mutations were generated by site-directed mutagenesis with 6 bp substitutions that changed 3 out of 9 bp of the nanomer and convert TRF site(s) to MluI recognition sequence. These mutants lacking TRF binding site at different degrees remain the same size as the plasmid containing intact TRF binding sites (Fig.4.6A).

To confirm that the oriP based plasmid and chimeric derivative were replicated in an EBNA1 depentdent fashion, we tested plasmid replication in the presence and absence of EBNA1 expression. Episomal DNA isolated by Hirt method at 4 days posttransfection was

subjected to Dpn I digestion overnight and subsequently detected for Dpn I-resistant DNA by Southern analysis. Newly synthesized DNA was observed in cells expressing EBNA1, 293E but not in 293 cells (Fig.4.6B). There are, although, differences in the levels of replication for each plasmid, which could be due to variations in transfection and DNA recovery efficiency, the results clearly demonstrat that oriP and chimeric origin plasmids were replicated in an EBNA1-dependent fashion.

To determine whether TRF bining sites contribute to plasmid retention, we performed a long-term replication assays in 293E cells to compare the stable plasmid maintenance of the hybrid origins containing wild type TRF binding sites with that of plasmids carrying mutated TRF binding site(s). Cells were transfected with plasmids, grown in the presence of 1 μg/ml puromycin for 4 days to select out plasmid containing cells and allowed to propagate under nonselective conditions for several rounds of cell division. During cultivation, equal numbers of cells were harvested at different time points to analyze for stable maintenance of the chimeric origin plasmids using Southern blotting with a radioactively labeled probe specificly against amplicilin gene. At 21 and 45 days after transfection, we were able to detect Dpn I-resistant DNA from plasmid containing wild type or mutated TRF binding sites. One, two, or all three mutations of the binding sites together have no demonstrable effect on plasmid maintenance. However, we observed changes in copy number conferred by TRF binding site mutants when compared to the wild type.

**DISCUSSION**

Many latent viruses such as PVs, EBV, and KSHV have evolved strategies to establish long-term persistence and maintain their genomes as episomal DNA in proliferating cells. To

ensure survival of their genomes in host cell nuclei, the viruses attach their genomes to mitotic chromosome for effective partitioning and thus prevent genome loss during mitosis. Considering efforts have been undertaken in the past years to define *cis* elements and *trans* acting factors required for such chromosomal tethering for different viruses. For papillomaviruses, the initial studies carried out in BPV1 have shown that the E2 protein plays a major role in the viral DNA maintenance by directing the genome to the mitotic chromosome via protein-protein interaction with cellular chromatin associated bromodomain protein (Brd4). From this, it was proposed for all PVs that E2 from different types can form a link between the viral genome and mitotic chromosomes, which allows equal distribution of newly replicated viral genomes to daughter cells. However, this mechanism has been further investigated in multiple human papillomaviruses with subtle disparities between genera reported. Although the segregation mechanism of the viral DNA via targeting mitotic chromosome has remained the common theme for all papillomaviruses, the *cis* elements and *trans* acting factors required for different papillomavirus types might be different.

In contrast to BPV1, HPV16 has less number of E2 binding sites than the minimal sites that are required for viral DNA maintenance. HPV16 E2 bound to mitotic chromosome with less efficiency and interaction occurred in different mitotic phase with unique mitotic localization pattern (Donaldson *et al.*, 2007, Oliveira et al., 2006). The dramatic disparity between BPV1 and HPV16 also existed in the mode of replication. While BPV1 replicated to yield high copy number in a random-choice manner (Piirsoo et al., 1996, Ravnan *et al.*, 1992), HPV16 DNA replicated at low copy number in an ordered once-per-S-phase manner (Hoffmann et al., 2006). These raise the question of whether viruses that maintain genomes at low copy number have a different target for genome tethering from those maintained at high copy number.

In previous experiments, we identified *cis* acting elements that were required for genome stability independently of E2 protein. These results support the concept that HPV16 E2 is not the only contributing factor to HPV DNA maintenance. By mapping studies, we found TRF binding sites present in the maintenance element in HPV16 and also track the existence of TRF binding sites mostly in the late region of all HPVs. Finally, the contribution of TRF2 to DNA maintenance evidenced in EBV led us hypothesized that TRF could play a role in HPV DNA maintenance.

Separate studies in high risk HPVs (types 31 and 16) reported that high risk E6 is necessary for episomal maintenance of HPV in primary keratinocytes. Genetic analyses suggested that p53 degradation activity of E6 is necessary for stable maintenance of high-risk HPV genome (Park and Androphy, 2002). Recently, E6 has been reported to interact with hTERT and bind to telomeric DNA possibly by TRFs suggesting a role in HPV replication or persistenance (Liu *et al.*, 2009). However, it was not clear whether other activity of E6 in telomerase stimulation would be relevant to the maintenance of high-risk HPV genomes. Since elevation of telomerase has an impact on expression profiles of telomeric related proteins (TRF1, TRF2, POT1, TIN2, TPP1, RAP1 and RecQ helicases), it is attractive to speculate that E6 facilitates the viral DNA maintenance by altering expression of these key factors. We therefore set out to determine the alteration of telomeric associated proteins levels in keratinocytes that have been immortalized by HPV16 DNA. Various transformed/immortalized cells as well as normal primary keratinocytes were also included. Compared to normal keratinocytes, TRF1, TRF2, and hTERT were expressed at higher levels in most transformed cells except osteoblastoma cells which are telomerase defective cells. Alteration of telomerase and telomere binding proteins levels has been previously reported in several kinds of cancers (Cookson and Laughton, 2009). However,

pattern of change seems to vary and depends on multiple factors such as cancer type, stage and genetic content. Involvement of TRF1 and TRF2 in telomere maintenance, recombination, replication, and DNA damage suggest a role in carcinogenesis. It is likely that elevation of TRF2 in HPV16 immortalized keratinocytes may be implicated in immortalization capability of HPV16. Recently, TRF2 was shown to be associated with latent origin replication of KSHV and EBV (Deng et al., 2003, Hu *et al.*, 2009). In EBV, TRF2 was also shown to stimulate oriP replication and contributed to episomal maintenance of oriP plasmid (Atanasiu et al., 2006, Deng et al., 2002). However, up-regulation of TRF2 in cells carrying HPV16 genome and its association with the binding sites within *cis*-maintenance element in HPV16 observed in this study questioning a role of TRF2 in the HPV16 DNA replication and maintenance.

By using ChIP assay, we could observe the interaction of TRF2 with three out of four putative TRF binding sites in HPV16 genome. Other telomere related proteins, POT1 and WRN were also associated with the same binding sites. POT1 and TRF2 were reported to bind telomeric repeats at the end of chromosomes and function in telomere maintenance (Court *et al.*, 2005, Gilson and Geli, 2007, Yang et al., 2005). They recruit WRN and stimulate helicase activity to unwind telomeric DNA (Ahn *et al.*, 2009, Opresko *et al.*, 2002). In contrast to TRF2 and POT1, WRN does not bind to DNA in a sequence-specific fashion suggesting that association of WRN with the TRF binding sites is mediated by protein-protein interaction with TRF2 or POT1. While TRF1 binds to identical sites as TRF2 (Bianchi *et al.*, 1999), we did not detect TRF1 or its binding partner TIN2 at the putative TRF binding sites. This result indicated that biological function of this binding site is specifically facilitated by TRF2 activity.

Although association of TRF2 on viral genome seems to be conserved among KHSV, EBV, and HPV, mutation of TRF2 binding sites did not affect HPV16 maintenance in long-term

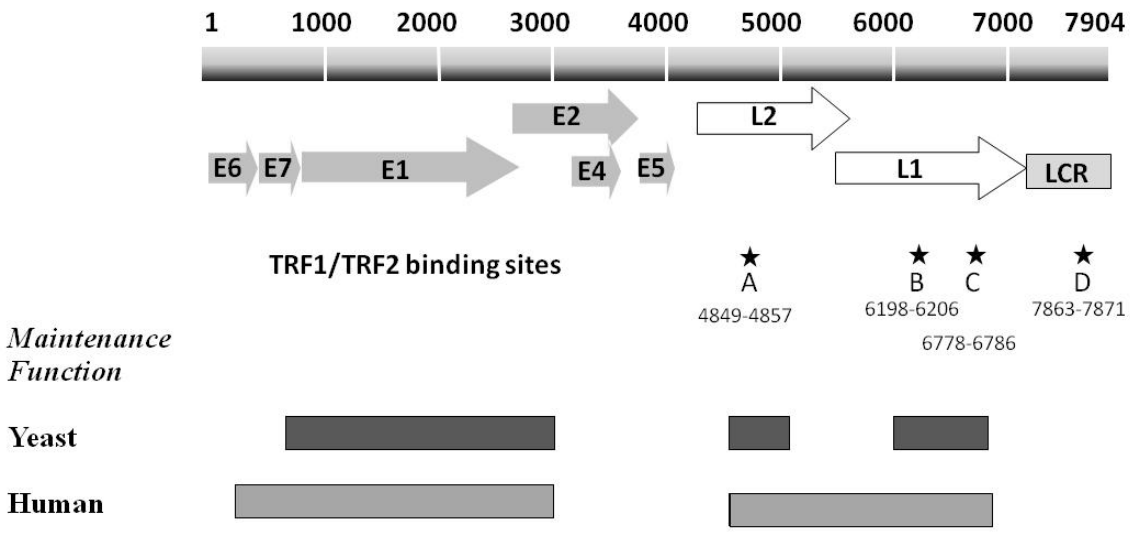
replication assay, suggesting additional roles of TRF2 on HPV16. In addition to protecting telomeres by forming an unusal structure with POT1, TRF2 is involved in DNA damage repair by relocating to double-stranded break sites with other DNA-repair proteins, Mre11-Rad50-Nbs1 (MRN) or ku70 (Song *et al.*, 2000). Thus, TRF2 behaves like a DNA damage sensor to controls genetic fidelity under genotoxic stress. A number of DNA damage repair proteins have been shown to be bound at latent origin of replication in KHSV (Skalsky et al., 2007)and EBV (Dheekollu *et al.*, 2007). Interestingly, WRN, a RecQ DNA helicase which is downstream of double strand break repair process was also found at the TRF binding sites on HPV16 genome in our study. Therefore it strongly suggests that TRF2 may perform other additional functions in HPV16 DNA maintenance in the condition of genotoxic stress. Recent study in EBV has also shown that MRN components was found at OriP and may act coordinately with TRF2 at the site to stimulate the recombination-like structure that provide sister chromatid cohesion through the topological linkages (Dheekollu et al., 2007). Resolution of these linkage leads to decatination of nascent episomal DNA that promotes genomes segregation and plasmid maintenance. HPV16 may adopt the similar mechanistic model for its genome maintenance. However, more investigations are required to detect the association of the MRN component on HPV16 genome as well as the formation of recombinant-like structure in order to address precise role of TRF2 in HPV16 life cycle.

In this study, we were not able to demonstrate implications of the TRF binding sites in plasmid retention by using TRF binding site mutants in a context of DS. The failure to observe influence of TRF binding site on HPV16 DNA maintenance may be due to the fact that there are other three intact TRF binding sites residing in the DS region. The robust origin activity of DS mediated by EBNA1 may compensate or mask the effect of mutated TRF binding site on DNA

maintenance as the plasmid stability was detected over 45 cell generations in the presence of DS alone (Figure 4.6, lane 2). The other possibility is that the binding of TRF2 on the sites might not be determined by DNA sequences since it was found on KHSV replication element that does not contain TRF consensus sequence (Skalsky et al., 2007). TRF2 was also found on the junctions of replication forks, chickenfoot structures, and Holliday junctions irrespective of the presence of consensus TTAGGG repeats (Fouche *et al.*, 2006). Therefore, mutation of the TRF binding sites may not completely abolish the TRF2 interaction that resulted in no noticeable differences in DNA retention activity between plasmid containing wild type versus mutated TRF binding sites. Additional experiments including the use of DN-TRF2, knocking-down of POT1 via siRNA, or a long-term replication assay in WS cells (WRN-/-) will allow us to examine the contribution of telomere binding proteins and its binding sites on HPV16 DNA maintenance.

HPV genomes are maintained as episomal DNA at a constant copy number in proliferating cells during persistent infection. The maintenance of the viral genome as an extrachromosomal plasmid requires an active mechanism of robust DNA segregation that prevents the catastrophic loss or damage of its genomic material. A number of proposed mechanisms for DNA maintenance in papillomavirus included multiple cellular proteins such as mitotic spindle (Van Tine et al., 2004), DNA helicase ChlR1 (Parish et al., 2006), RNA polymerase I transcription factor UBF (Poddar et al., 2009), and mitotic kinesin like protein (Yu *et al.*, 2007). In this study, we found interesting cellular taget, telomere binding factors (TRF2, POT1, and WRN) whose interaction was shown to be conserved and important for the DNA maintenance and genomic stability in other persistent episomal viruses, like KHSV and EBV. Although the implication of this interaction in HPV16 life cycle requires further characterization, the telomere binding factors could be attractive candidates for the development of antiviral therapy.

Figure 4.1 Telomere-related factor (TRF) binding sites in HPV 16 genome. HPV16 genome organization map showing 8 ORFs and the control region, LCR. The bars below represent maintenance elements mapped in human and yeast system. Putative telomere-related factor (TRF) binding sites, designated by A, B, C, and D located on HPV16 genome at indicated nucleotide positions. The bold letters indicate the mismatch nucleotides in predicted binding sites. Three of the TRF binding sites lie within the regions having maintenance activity in the L1 and L2 ORFs. The consensus TRF binding site and corresponding HPV16 sequence are shown below the map.




TRF1/TRF2 binding site consensus = TTAGGGTTA

A TTAGGGTT**T**

B TTAG**A**GTTA

C TTA**A**GGTTA

D TT**T**GGGTTA

Figure 4.2 Southern blot analysis of HPV16-immortalized foreskin keratinocytes (HPV16-NHFK) and levels of telomere repeat proteins expression in various cell types. A) HPV16-NHFK contains low copy number of HPV16 genome as an episomal DNA. Episomal DNA was extracted by Hirt method from foreskin kerationcytes that were transfected with the plasmid containing an entire HPV16 genome. After transfection, cells were continually grown beyond cell senescense crisis (> 30 passages) and become immortalized. The Hirt-DNA was linearized with a single-cutting enzyme, HindIII and HPV16 DNA was detected by southern blot using an HPV16 whole genomic probe labeled with radioactive $^{32}P$. As a copy number control, linealized pEF399 at concentration of 25, 2.5, and 0.25 copies per cell were loaded on the left of the blot. B) Western blot analysis of the expression of telomere related proteins in transformed and normal cells. Whole cell extracts were prepared from 4 day-old cells using SDS lysis buffer. Equal amount of protein extract (30 μg) was subjected to SDS-PAGE electrophoresis and then hybridized with specific antibody for telomere repeat proteins. The left panel demonstrated the expression levels of telomere related proteins (TRF1, TRF2, POT1, RAP1, hTERT, TIN2, and WRN) in cells transformed either spontaneously or virally compared to normal primary cells (left panel). Coomassie blue staining is included as a control for protein loading. U2OS (h-TERT defective osteoblast cells), U2OS 16E2 (U2OS expressing HPV16 E2 protein), HeLa (HPV18 transformed cervical cell line), HaCat (spontaneously transformed human keratinocyte cells), 293 (sheared adenovirus 5 transformed human embryonic kidney cell), 293T (293 expressing SV40 Tag), NHFK (neonatal human foreskin keratinocyte), and HFK (human foreskin keratinocyte). The right panel exhibited the expression levels of telomere related protein (TRF2, TIN2, POT1, RAP1, and WRN) in immortal foreskin keratinocytes compared to parental normal counterpart. HPV16-NHFK (HPV16 transformed neonatal human foreskin keratinocyte cells), and hTERT-HFK (hTERT immortalized human foreskin keratinocyte cells).

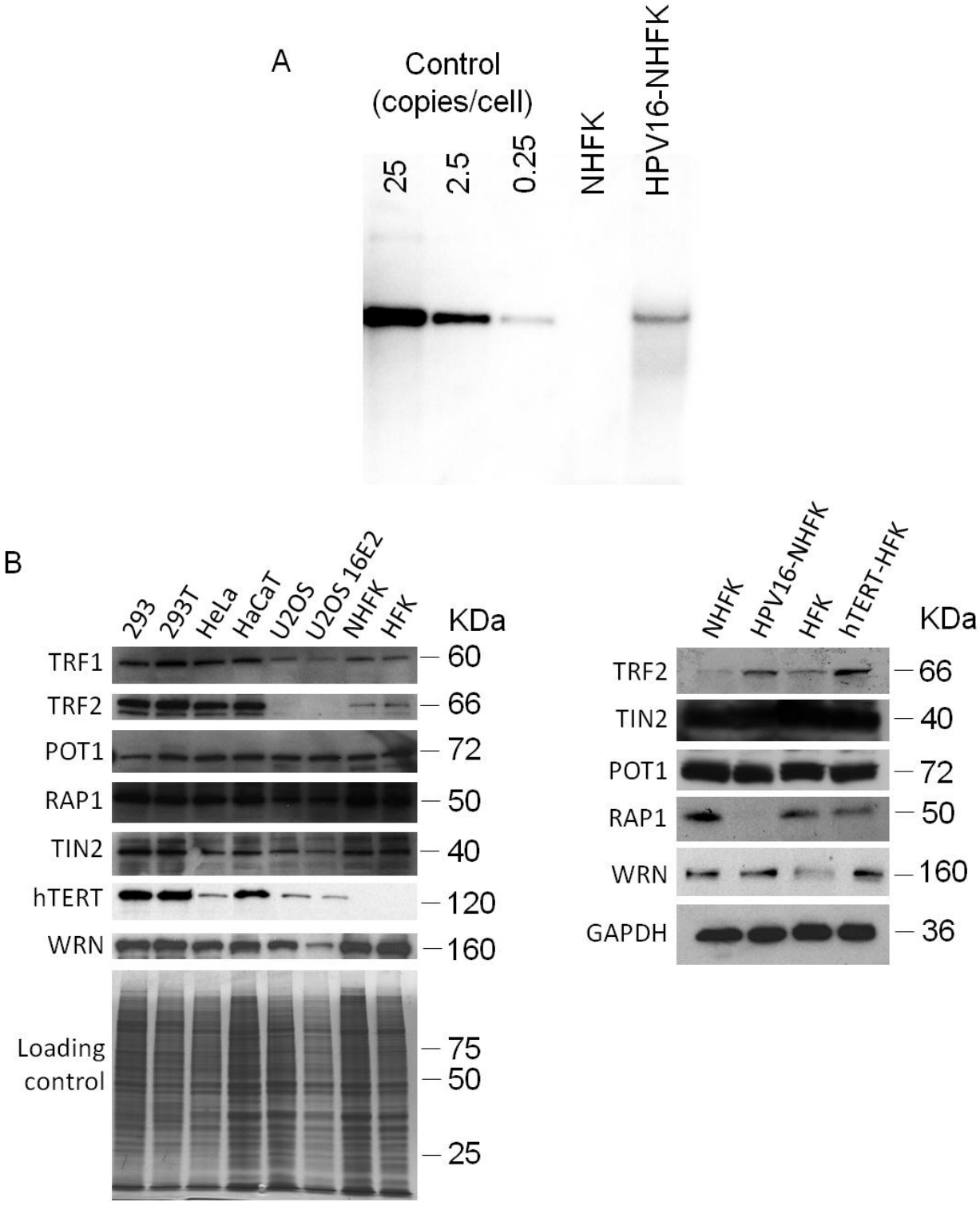
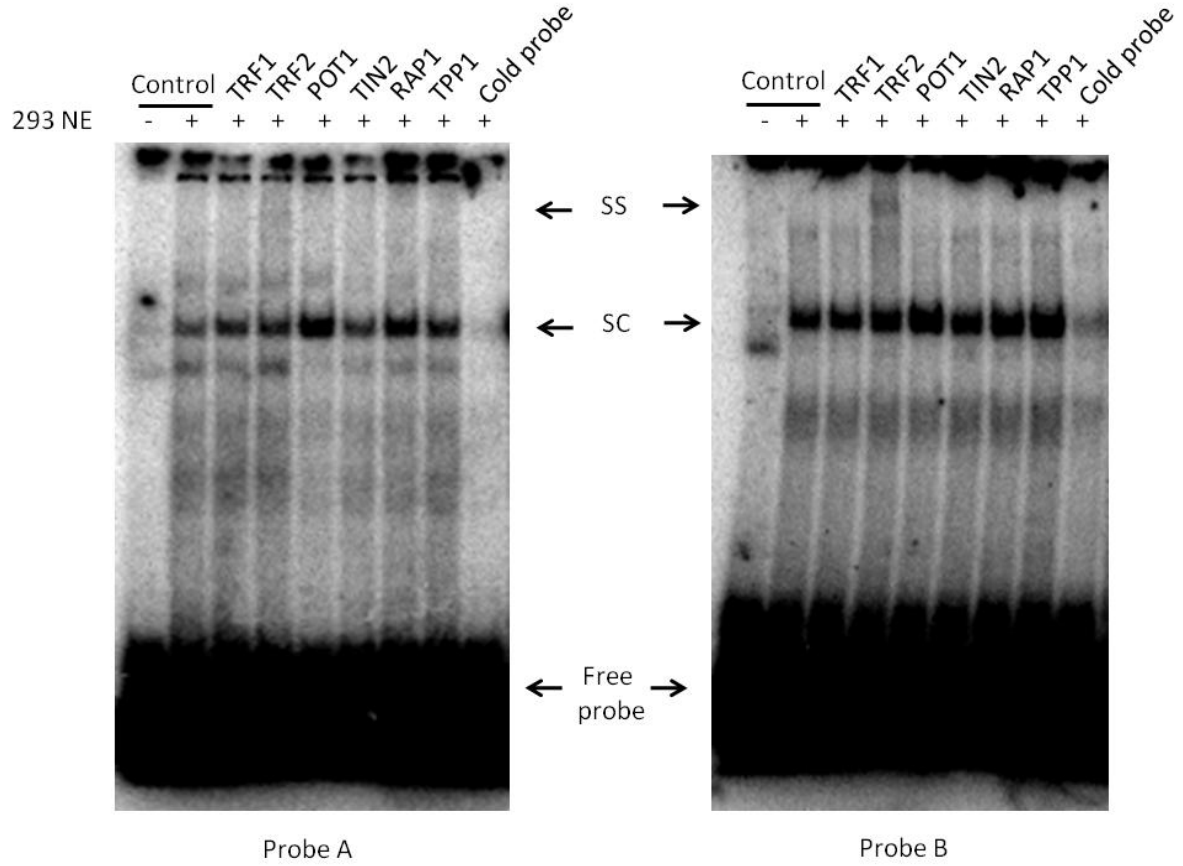

A
Control
(copies/cell)
25
2.5
0.25
NHFK
HPV16-NHFK
B
293
293T
HeLa
HaCaT
U2OS
U2OS 16E2
NHFK
HFK
KDa
TRF1
60
TRF2
66
POT1
72
RAP1
50
TIN2
40
hTERT
120
WRN
160
Loading control
75
50
25
NHFK
HPV16-NHFK
HFK
hTERT-HFK
KDa
TRF2
66
TIN2
40
POT1
72
RAP1
50
WRN
160
GAPDH
36

Figure 4.3 Supershift analysis for the interaction of telomere related proteins and TRF binding sequences in the HPV16 genome. Gel shift performed using 293 nuclear extract with $^{32}P$ labeled oligonucleotide probe harboring a predicted TRF binding site (denoted A, B, C, and D as shown in Fig 4.1 and described in Materials and Methods) as indicated. Antibodies specific for indicated telomere related proteins were used for supershift assays. The positions of free probe, shifted complex (SC) and supershift (SS) are indicated. Unlabeled oligonucleotides (cold probe) at concentration of 1.25 μM (100X) were used as a control to decrease intensity of the specific shifted bands.


293 NE
Control
TRF1
TRF2
POT1
TIN2
RAP1
TPP1
Cold probe
- + + + + + + + +
SS
SC
Free probe
Probe A
Probe B

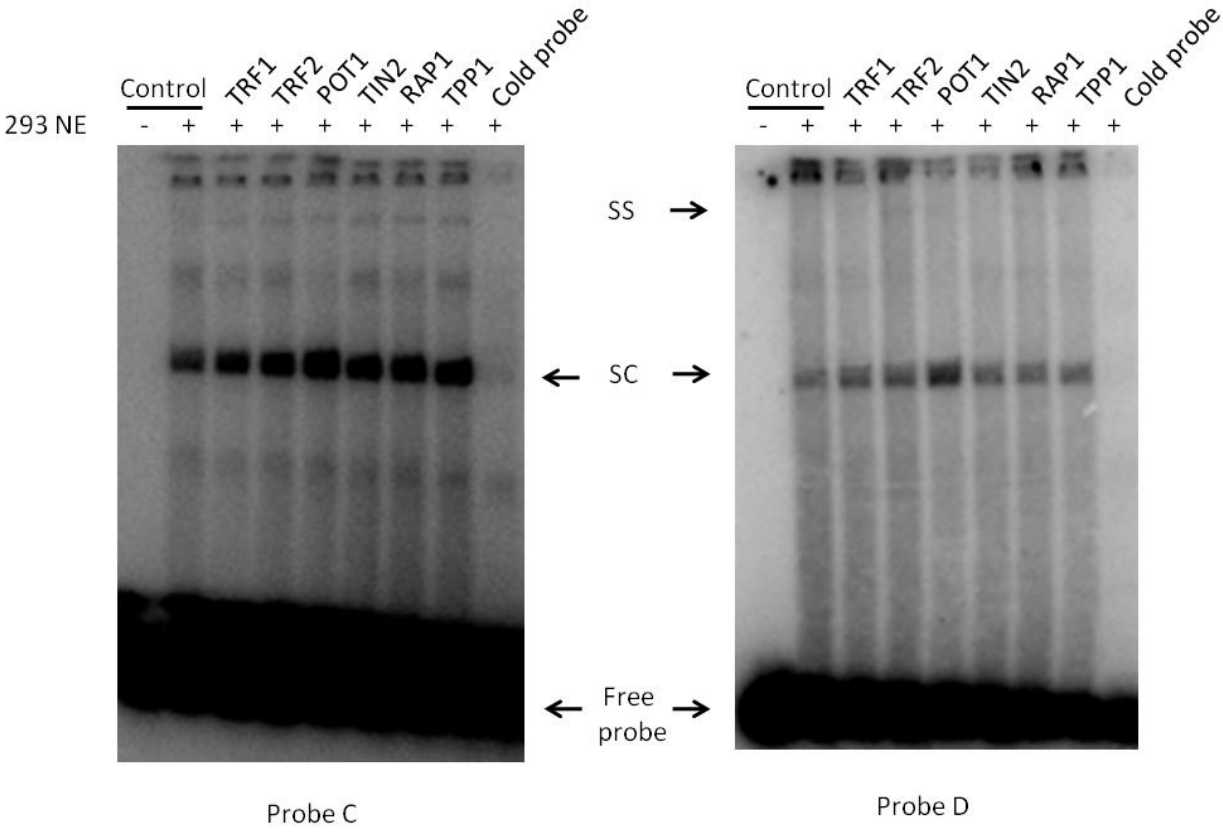
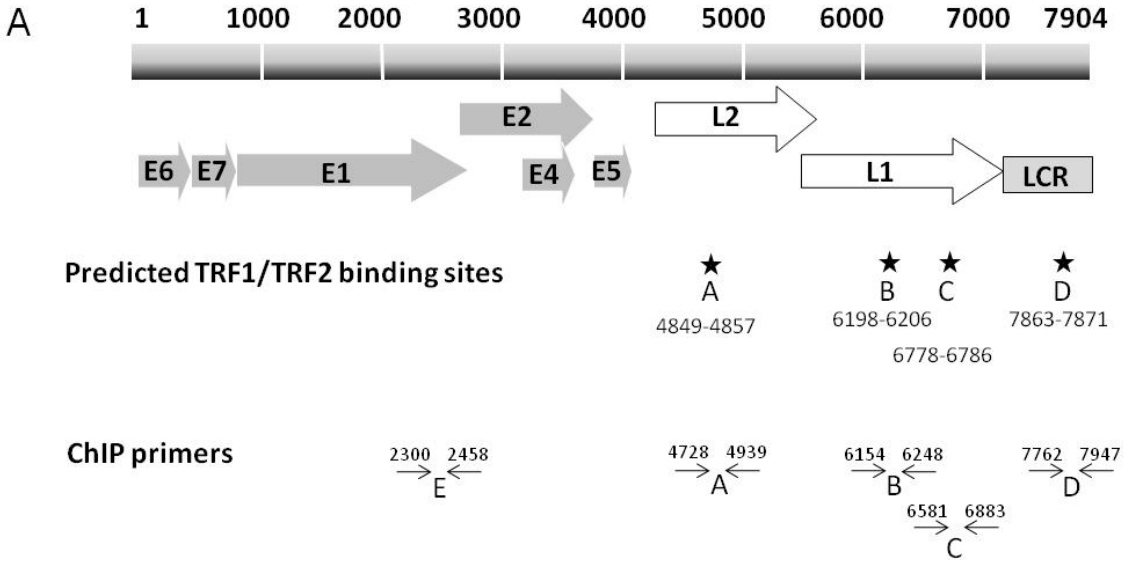

293 NE
Control
TRF1
TRF2
POT1
TIN2
RAP1
TPP1
Cold probe
- + + + + + + + +
SS
SC
Free probe
Probe C
Probe D

Figure 4.4 Binding of TRF2, POT1, and WRN to TRF binding sites in cells. A) Schematic representation of positions of putative TRF binding sites and primers used for PCR amplification. B) Average size of sheared DNA obtained from 293 cells transfected with pEF399 before subjection to immunoprecipitation. On the left lane, a 100-bp DNA ladder was used as a DNA size marker. C) ChIP analysis in 293 cells transfected with pEF399 which contains an entire HPV16 genome. At 4 days after transfection, cells were harvested, crosslinked, and subjected to ChIP assay with antibodies specific for telomere related proteins, no antibody or control IgG. Immunoprecipitated DNA was amplified with primers specific for TRF binding sites or control E2 region of the HPV16 genome. Mock transfected cells are used as a negative control in lane 19. ChIP values were quantified from at least 2 independent expereiments and presented as percentage of input DNA in the bar graph to the bottom panel. D) ChiP analysis in HPV16-NHFK. Crosslinked protein-DNA complexes were immunoprecipitated with specific antibodies to telomere related protein, control mouse IgG, or no antibody as described above. Immunoprecipitated DNA was amplified with primers specific for TRF binding sites A and D. Normal counterpart, NHFK cells were used as a negative control in lanes 1 and 2.


A
1
1000
2000
3000
4000
5000
6000
7000
7904
E2
L2
E6
E7
E1
E4
E5
L1
LCR
Predicted TRF1/TRF2 binding sites
A
4849-4857
B
C
6198-6206
6778-6786
D
7863-7871
ChIP primers
2300
2458
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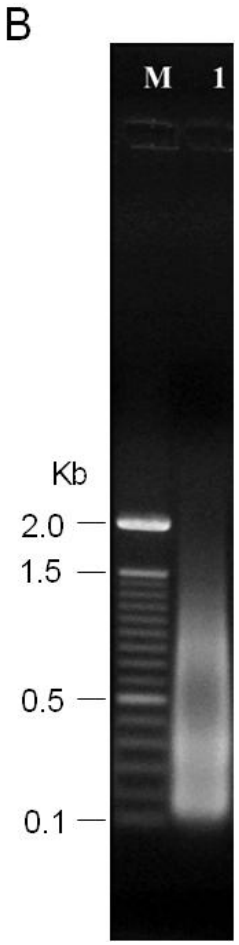
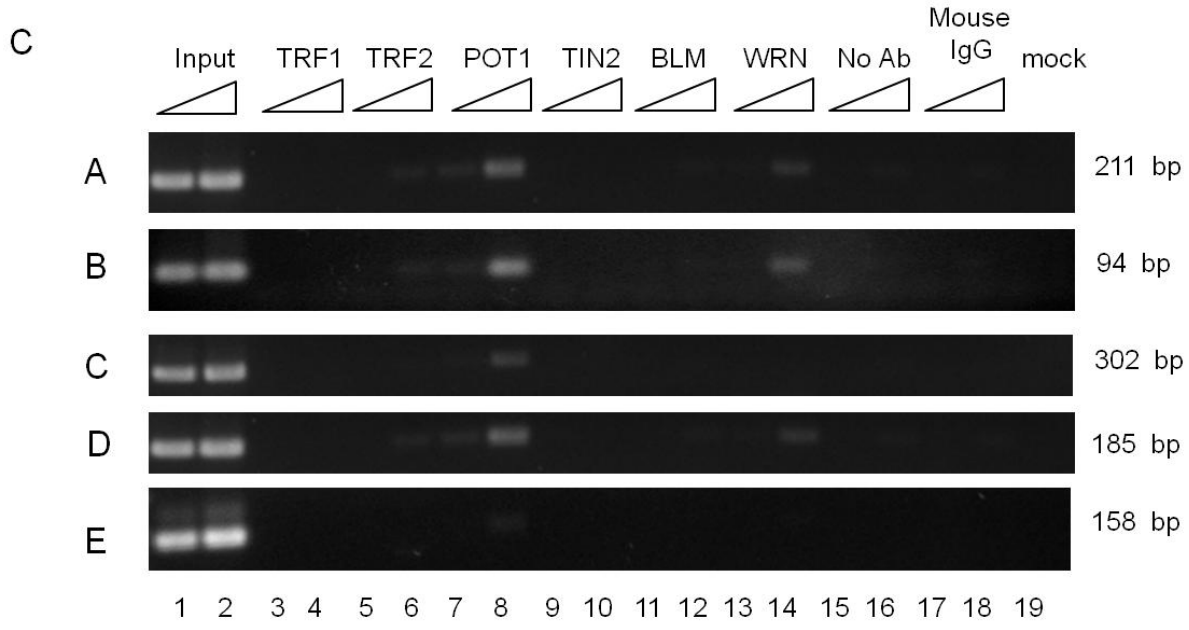
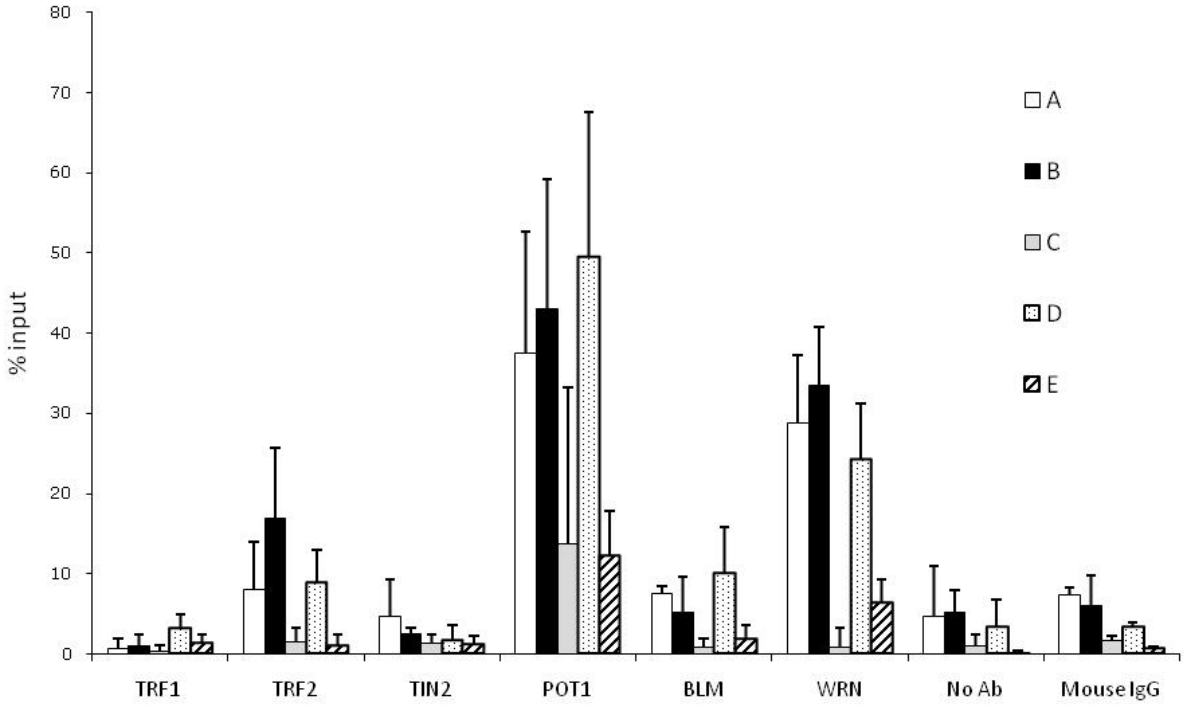
6248
B
6581
6883
C
7762
7947
D


B
M
1
Kb
2.0
1.5
0.5
0.1


C
Input
TRF1
TRF2
POT1
TIN2
BLM
WRN
No Ab
Mouse IgG
mock
A
B
C
D
E
211 bp
94 bp
302 bp
185 bp
158 bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19


% input
80
70
60
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A
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C
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E
TRF1
TRF2
TIN2
POT1
BLM
WRN
No Ab
Mouse IgG

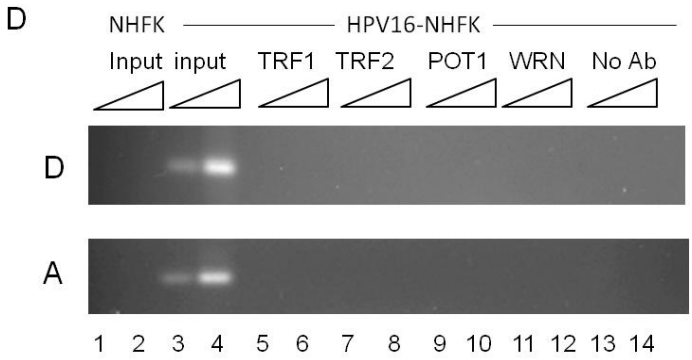
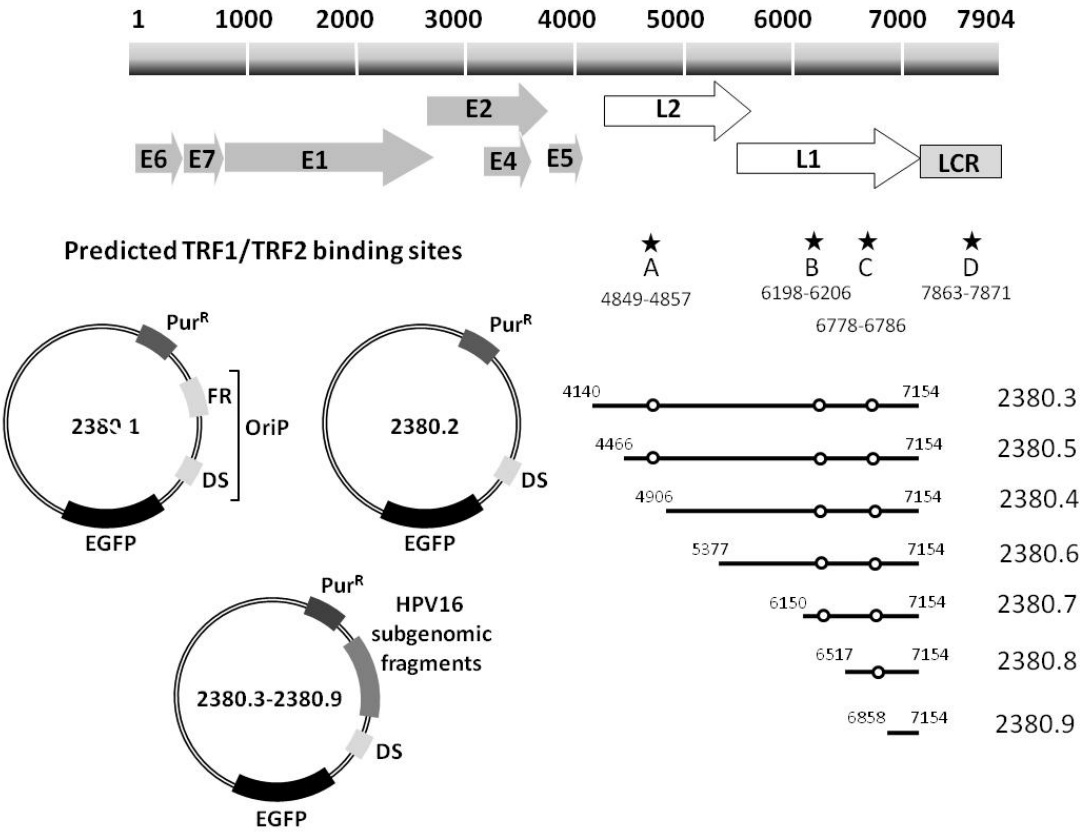

D
NHFK
HPV16-NHFK
Input
input
TRF1
TRF2
POT1
WRN
No Ab
D
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4.5 DNA stability of plasmids containing HPV16 subgenomic fragment with varied numbers of TRF binding sites. A) Schematic representation of HPV16 genomes organization with positions of putative TRF binding sites indicated as A, B, C, and D. Plasmid maps shown on the left represented EBV oriP-based plasmids used to examine influence of TRF binding site on HPV16 DNA maintenance. The plasmid 2380.1 contains a puromycin-resistance gene, an EGFP gene and an intact oriP of EBV comprising DS and FR. The FR sequence in 2380.1 was deleted to create 2380.2. Plasmids 2380.3-2380.9 were constructed as described in Materials and Methods by substraction of FR sequence with HPV16 subgenomic fragments shown on the right. B) The stability of plasmids containing HPV16 subgenomic fragments was demonstrated as the relative loss rate per cell generation. The percent of EGFP-positive cells was obtained by flow cytometry analysis at different time points. The change in percent EGFP positive cells per cell generation, presented in bar graphs as loss rate was calculated relative to the initial time point of each transfection under nonselective conditions. Results are shown as means $\pm$ standard error of two independent experiments.

A


1
1000
2000
3000
4000
5000
6000
7000
7904
E2
L2
E6
E7
E1
E4
E5
L1
LCR
Predicted TRF1/TRF2 binding sites
A
4849-4857
B
6198-6206
C
6778-6786
D
7863-7871
Pur^R
FR
OriP
DS
EGFP
2380.1
2380.2
Pur^R
DS
EGFP
Pur^R
HPV16
subgenomic
fragments
2380.3-2380.9
DS
EGFP
4140
7154
2380.3
4466
7154
2380.5
4906
7154
2380.4
5377
7154
2380.6
6150
7154
2380.7
6517
7154
2380.8
6858
7154
2380.9

B

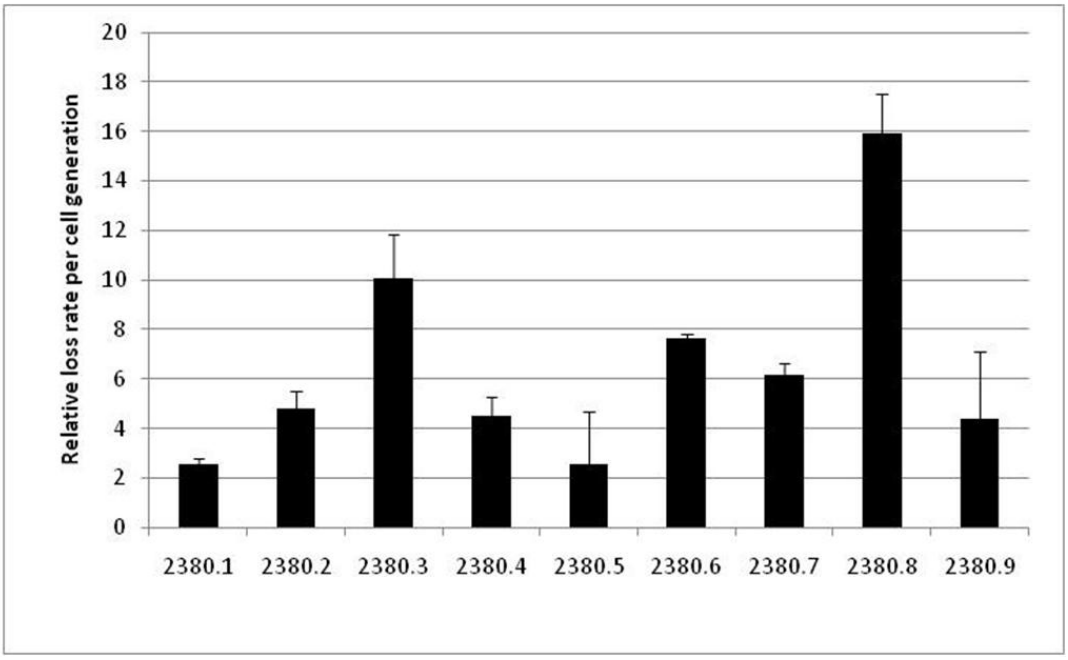
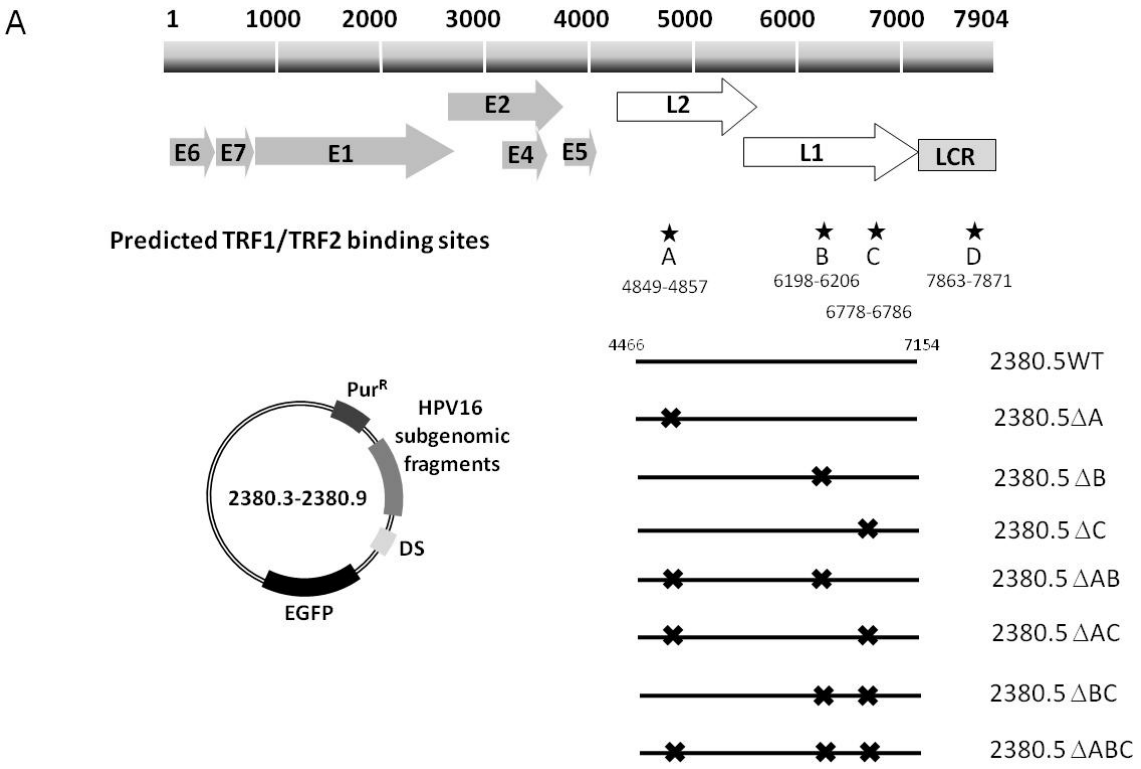
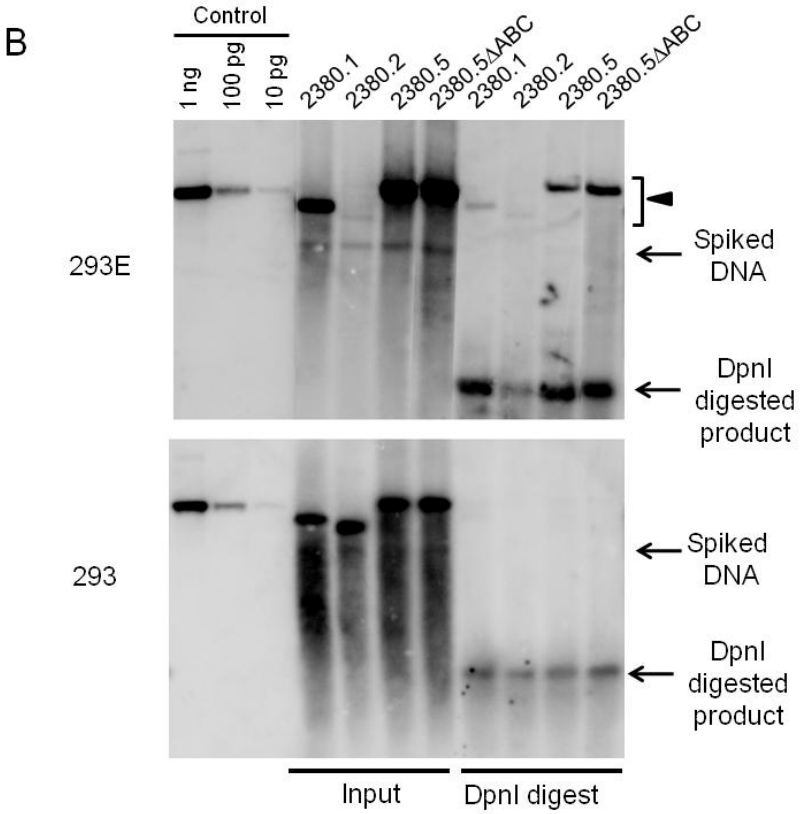
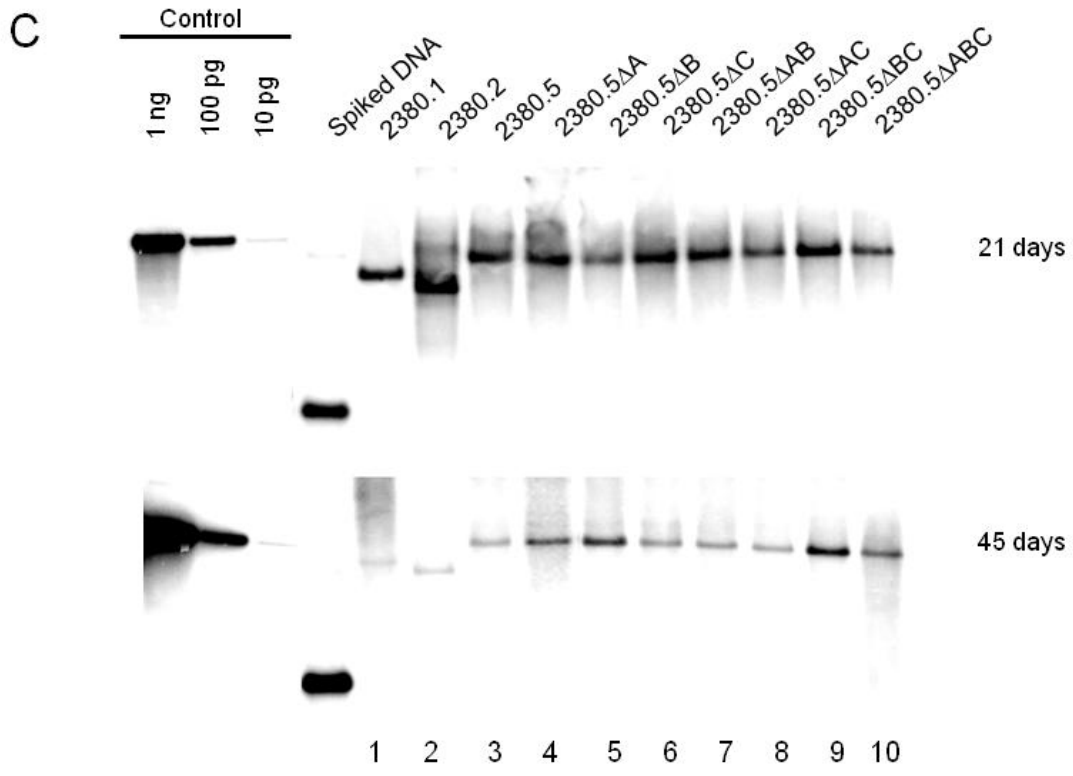

Relative loss rate per cell generation
20
18
16
14
12
10
8
6
4
2
0
2380.1
2380.2
2380.3
2380.4
2380.5
2380.6
2380.7
2380.8
2380.9

Figure 4.6 Analysis for a role of TRF binding sites in HPV16 DNA maintenance. A) Schematic represention of plasmid 2380.5 containing EBV dyad symmestry (DS), a puromycin-resistance gene, an EGFP gene and the late region of HPV16 DNA (nt 4466-7154) with TRF binding sites (A, B, and C). A series of mutant constructs containing single, double or triple mutations at specific TRF binding sites were made by converting the TRF binding sites in the plasmid 2380.5 to Mlu I recognition sequences using a site-directed mutagenesis kit (Stretragene). The point mutations were illustrated as "x" and designated as indicated in the diagram. B) Transient replication of oriP based plasmids in 293E (top panel) or 293 (lower panel) cells. Short-term replication assay was performed as described in Material and Methods. The arrowhead to the right of the top panel shows the newly replicated DNA. Control DNA, loaded on the left contains 1 ng, 100 pg, and 10 pg of linearized 2380.5. C) Stable maintenance properties of OriP chimeric plasmids containing HPV16 subgenomic fragments in 293E cells. As described in Materials and Methods, cells were transfected and subsequently subjected to puromycin treatment for 4 days to select for plasmid containing cells. Episomal DNA was Hirt-extracted at 21 and 45 days after transfection under nonselective conditions. Prior to DNA isolation, each sample was spiked with 10 ng of pUC19 in order to monitor the completeness of Dpn I digestion. Ten percent of the purified DNA was linerized with Xcm I as input, and the remaining was double digested with Xcm I and Dpn I. Newly synthesized, Dpn I resistant DNA was detected by Southern blotting using radiolabeled amplicilin specific probe. The position of bacterially synthesized pUC19 DNA added to the sample (spiked DNA) is shown by an arrow to the left of the blots. Linearized 2380.5 at indicated concentrations was loaded on the left and used as a control DNA.


A
1
1000
2000
3000
4000
5000
6000
7000
7904
E2
L2
E6
E7
E1
E4
E5
L1
LCR
Predicted TRF1/TRF2 binding sites
A
4849-4857
B
6198-6206
C
6778-6786
D
7863-7871
4466
7154
2380.5WT
2380.5ΔA
2380.5 ΔB
2380.5 ΔC
2380.5 ΔAB
2380.5 ΔAC
2380.5 ΔBC
2380.5 ΔABC
Pur^R
HPV16
subgenomic
fragments
2380.3-2380.9
DS
EGFP


B
Control
1 ng
100 pg
10 pg
2380.1
2380.2
2380.5
2380.5ΔABC
2380.1
2380.2
2380.5
2380.5ΔABC
293E
Spiked
DNA
DpnI
digested
product
293
Spiked
DNA
DpnI
digested
product
Input
DpnI digest


C
Control
1 ng
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Spiked DNA
2380.1
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2380.5ΔB
2380.5ΔC
2380.5ΔAB
2380.5ΔAC
2380.5ΔBC
2380.5ΔABC
21 days
45 days
1 2 3 4 5 6 7 8 9 10

## General Conclusions

The work presented in this dissertation is focused on identification of *cis*-elements and *trans*-acting factors that contribute to HPV16 DNA maintenance in mammalian cells during persistent infection. Considerable efforts have been undertaken in the past years to elucidate the mechanisms underlying the papillomaviral genome maintenance. Extensive studies using BPV1 have revealed that the virus makes use of the viral E2 protein to link its genomes to mitotic chromosome via interaction with a cellular chromatin-associated protein, Brd4, in order to equally segregate its genomes to new daughter cells. However, the mechanism found in BPV1 does not apply to HPV due to subtle disparities between genera. It was noted that HPV E2 is not able to bind to mitotic chromosome as tightly as BPV E2 and Brd4 is not required for the maintenance of HPV genome. Furthermore, by using a yeast system, HPV16 has been shown to stably replicate and maintain its genomes independently of E2. Although the segregation of viral genomes through an association with host mitotic chromosome remains the common theme for papillomavirues, HPV may target different cellular factors to accomplish the long-term DNA maintenance in infected cells. Understanding of how HPV replicate and maintain their genomes during persistent infection will shed light on mechanism and cellular targets that the virus used in this process, giving us invaluable information for future development of potential antiviral agents.

Previous experiments in yeast demonstrated that HPV16 can replicate in the absence of any virally encoded replication proteins, E1 and E2. In agreement with this observation, Hoffmann et al. reported that HPV16 replicated like the cellular DNA in a once per cell cycle fashion, indicating that the viral replication is greatly rely on cellular replication factors. In this study, we performed a short-term replication assay in mammalian cells using a series of HPV16

deletion mutants to demonstrate that HPV16 can replicate independently of trans-viral factors, E1 and E2. The results clearly supported that HPV16 replication entirely relies on host replication factors. Further investigation, under conditions without viral E1 and E2 proteins, found that HPV16 subgenomic fragments lying within the E7 ORF (nt 622-870) and late region (nt 5072-6905) could substitute for human autonomously replicating sequence (hARS) suggesting that HPV16 utilizes distinct origins to initiate replication in the E1-E2 independent mode of replication.

We also demonstrated that two distinct regions in HPV16 genome provide capability of DNA stability in host cells in the absence of any viral protein expression. These subgenomic fragments are located in the early (nt 525-3479) and late regions (nt 4538-6950) and they are overlapping with the sequences that have been mapped as maintenance elements in yeast. The *cis*-maintenance element in the late region contains putative binding sites (TTAGGGTTA) for telomere binding proteins (TBPs) that have been found on EBV latent origin of replication and contributed to episomal EBV maintenance. In addition to the putative binding sites within the HPV16 DNA, we also found altered expression levels of TBPs in HPV16 immortalized keratinocytes. These findings provided a possibility that HPV16 may have evolve similar mechanism in EBV to maintain its genome in host cells.

In this study, a number of proteins that associate with telomeric repeats were examined whether these proteins would bind to the putative TRF binding sequences. By ChIP assay, the telomere binding proteins, TRF2, POT1, and a DNA helicase WRN were detected on the predicted TRF binding sites in HPV16 containing cells. Depletion of TRF binding sites by deletion have shown influences on plasmids stability in a long-term assay using flow cytometry analysis. However, we could not clearly demonstrate the effect of this interaction on DNA stability when

we used mutants carrying point mutions in TRF binding sequences by Southern analysis. It was possibly due to limitation of experimental conditions. Long-term replication assay performed by using a dominant negative mutant protein or TBP knock-out cells should be an alternative way to determine implications of this interaction on episomal DNA maintenance. Since these telomere binding proteins have additional functions in other important pathways such as homologous recombination and DNA damage repair, it is likely that TBPs may play additional roles in HPV16 life cycle under genotoxic stress. The overlapping of autonomously replicating sequences and an association with the WRN helicase found on this maintenance element suggest that these telomere-related proteins could possibly play a role in replication. Further experiments are required to determine the exact role of these proteins in HPV life cycle. Furthermore, multiple binding sites for TopoII, CENP-B, and HMG predicted within the late region of HPV16 further suggests that the HPV maintenance mechanism is influenced by more than one cellular factor.

Our findings in this study provide additional information and give us more insight into HPV DNA maintenance during persistent infection. The search for elements involved in this process has been of long-standing interest in the field. An understanding of interactions required for establishment and maintainance of the viral genome provides informative insight to design attractive drug targets. Novel antiviral therapeutics that disrupts the viral genome maintenance during persistent infection would inhibit progression of tumorigenesis. Papillomaviruse-based plasmid is also an attractive choice for use in gene therapy because it can replicate extrachromosomally and the small size is easy to manipulate. A more detailed knowledge of the mechanisms of HPV maintenance will enable us to develop powerful vectors with minimal potential to cause complications in gene therapy treatment.

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